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EARLY DIETARY SOY PROTEIN ATTENUATES RENAL DISEASE AND ALTERS RENAL AND HEPATIC FATTY ACID COMPOSITION IN WEANLING HAN:SPRD-*cy* RATS

BY

DENISE ELIZABETH FAIR

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for a Degree of

MASTER OF SCIENCE

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree of MASTER OF SCIENCE

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ABSTRACT

Soy protein slows disease progression and modifies fatty acid status in kidney and liver tissues after 6 weeks of feeding in the Han:SPRD-*cy* rat model of polycystic kidney disease. To determine whether early dietary soy protein feeding alters the progression of chronic renal disease, renal and hepatic fatty acid status, and renal ex vivo release of PGE_2, three-week-old heterozygous male Han:SPRD-*cy* rats (n=87) were given 20% casein or soy protein based diets for 1 or 3 weeks. Immunohistochemical analysis revealed that soy feeding reduced fibrous volume after 1 week of feeding and renal cyst volume after 3 weeks of feeding in diseased animals. Fatty acid analysis revealed that soy feeding elevated kidney linoleic acid (weight %) in both normal and diseased animals at 1 week (15.81 ± 0.25% vs 12.16 ± 0.24%, $P<0.05$) but not at 3 weeks. Lower renal arachidonic acid was observed at 3 weeks in diseased animals (24.32 ± 0.87 % vs 27.77 ± 1.05 %, $P< 0.05$) compared to normals, with no effect of diet observed. Similar to the kidney, hepatic linoleic acid was elevated by dietary soy protein at 1 week (17.34 ± 0.26 % vs 13.82 ± 0.27 %, $P<0.05$) but not at 3 weeks. Hepatic arachidonic acid content was higher overall in soy fed animals when compared to casein fed animals (19.50 ± 0.34 % vs 17.74 ± 0.34 %, $P<0.05$). Whole tissue ex vivo release of prostaglandin E_2 (nmol/kidney) doubled in diseased animals from 1 to 3 weeks of feeding while normal animals remained consistent over time. Soy protein alters renal fibrous volume and renal and hepatic fatty acid composition after 1 week of dietary intervention. After only 3 weeks of feeding, soy demonstrates an overall attenuation of early renal disease progression, emphasizing the importance of early dietary intervention in this disease.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ADPKD	autosomal dominant polycystic kidney disease
ARPKD	autosomal recessive polycystic kidney disease
ATPase	adenosine triphosphatase
COX	cyclooxygenase
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EFA	essential fatty acid
EGF	epidermal growth factor
EPA	eicosapentanoic acid
ESRD	end-stage renal disease
GC	gas chromatography
GFR	glomerular filtration rate
HPLC	high-performance liquid chromatography
LA	linoleic acid
LNA	α-linolenic acid
MUFA	monounsaturated fatty acids
NO	nitric oxide
PG	prostaglandin
PGE_2	prostaglandin E_2
PKD	polycystic kidney disease
PLA_2	phospholipase A_2
PTH	parathyroid hormone
PUFA	polyunsaturated fatty acids
SAF	saturated fatty acids
SEM	standard error of mean

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1 Rationale

As many as 60 000 Canadians and 600 000 Americans live with polycystic kidney disease (PKD) (as cited in O'Neill, 1999). It affects all races, ethnicities, ages and both sexes and leads to end stage renal failure in over half of its victims by the age of 60 (Gabow, 1993). This disease greatly reduces the quality of life for the individuals faced with it and costs 1.5 billion dollars to treat (as cited in Zak, 1997). As of yet, there is no cure for PKD.

Although PKD usually manifests around the fifth or sixth decade of life, this disease is an infantile disease as PKD alters renal cell structure during early development (McCarthey and McMullen, 1997). Current treatment of renal disease focuses on treating the end stage renal failure and the complications that arise. However, research conducted on preventative therapy as a treatment for renal disease has found that intervention prior to end stage renal disease alters disease progression and can prolong longevity (Maschio et al, 1983)

Protein restriction has been recommended in renal insufficiency for over a century (as cited in Velasquez and Bhathena, 2001). The notion that modification of dietary protein could alter renal disease progression has recently been addressed and has great implications. Both the quality and quantity of protein ingested can influence disease progression. Low protein diets and soy protein cause less structural deterioration, preserves renal function and slows cyst development and disease progression (Williams et al, 1987; Aukema et al, 1992; Tomobe et al, 1994; Ogborn et al, 1995; Ogborn et al, 1998; Aukema et al, 1999; Aukema et al, 2001). However, dietary protein intervention

has little effect on later stages of renal insufficiency (Locatelli et al, 1991) stressing the importance of early dietary intervention.

Although in animal models soy does attenuate the progression of PKD (Tomobe et al, 1998; Aukema et al, 1999; Ogborn et al, 1998), the exact mechanism of how soy exhibits its effects is still under investigation. Ogborn and colleagues (2000) discovered that soy protein feeding alters the fatty acid status of renal and hepatic tissues, suggesting that soy protein may alter polyunsaturated fatty acid (PUFA) metabolism. Therefore this thesis research was designed to further current knowledge on how soy alters cyst development and disease progression by examining fatty acid composition in renal and hepatic tissues and to examine the importance of early soy protein intervention on renal disease progression.

Hypotheses: soy protein feeding to weanling Han:SPRD-*cy* rats in early renal disease will maintain renal function and delay cyst development and disease progression after 1 and 3 weeks of feeding. Soy protein will alter polyunsaturated fatty acid metabolism and modify fatty acid status in both renal and hepatic tissues and will alter kidney prostaglandin E_2 (PGE_2) production.

The **objectives** of this research are to determine whether dietary soy protein, after as early as 1 to 3 weeks of feeding in weanling Han:SPRD-*cy* rats will alter:

1) cyst development and disease progression of chronic renal disease
2) hepatic and renal fatty acid compositions as seen previously after 6 weeks of feeding
3) eicosanoid metabolism by quantifying renal PGE_2 ex vivo release

2 Present Stage of Knowledge

2.1 Polycystic Kidney Disease (PKD)

PKD is an inherited genetic disease that is characterized by cyst formation in ductal organs, particularly in the kidney and liver (Gabow, 1998). It is the most common renal genetic disease, affecting more people than cystic fibrosis, muscular dystrophy, hemophilia, Down's Syndrome and sickle cell anemia combined (Zak, 1999). Although the prevalence of this disease is twenty times more frequent than cystic fibrosis it is relatively unknown in the general public (McCarthy and McMullen, 1997). PKD affects people of all ages, races, ethnicities, social status and sex (Zak, 1999). It is the fourth most common cause of end stage renal disease (ESRD), accounting for 8–10 % of all cases (Gabow, 1993). For these people, dialysis or kidney transplantation is necessary for survival with the cost of treatment exceeding billions of dollars a year in the United States (Zak, 1997).

There are at least two different types of polycystic kidney disease: autosomal dominant polycystic kidney disease (ADPKD), which may represent many genetic disorders with a similar phenotype and autosomal recessive polycystic kidney disease (ARPKD). They have comparable pathophysiology and etiology and both can lead to ESRD. ARPKD, also frequently called infantile PKD, has an incidence of 1 in 6 000 to 1 in 40 000 people in North America (Murcia et al, 1998). It typically manifests early in life, usually *in utero* and is the more severe form of PKD. The high incidence of infantile death in ARPKD can be attributed to a reduction in fetal renal output causing lower levels of amniotic fluid *in utero* that hinders fetal development. Infants are often born with pulmonary hypoplasia (small lungs) and consequently, 30-50% die at birth or soon

after due to respiratory failure (Guay-Woodfordf et al, 1996). Of the infants that survive the newborn period, an estimated one third will require dialysis or transplantation by the age of ten (Guay-Woodford et al, 1996) and 78% will survive beyond the age of 15 (Roy et al, 1997).

ADPKD occurs more frequently, affecting 1 in 500 to 1 in 1000 (Murcia et al, 1998) and is often referred to as the "adult" form of PKD. Symptoms usually manifest in the fifth to sixth decade of life, although it can affect all ages (McCarthy and McMullen, 1997). Not all individuals with ADPKD exhibit symptoms of renal insufficiency and some affected individuals remain asymptomatic for life with a diagnosis being made upon autopsy. Approximately 50% of ADPKD patients will progress to end stage renal disease before the age of 60 (Gabow, 1993).

Diagnosis of all types of ADPKD is not difficult once the disease is fully developed, as kidneys are bilaterally enlarged and have irregular surfaces that can be detected upon palpation (Welling and Grantham, 1996). DNA linkage studies are commonly used to diagnose ADPKD when a positive family history is present (Gabow, 1990). ARPKD is commonly diagnosed after birth but can be detected *in utero* after 24 weeks of gestation by evidence of large hyperechoic kidneys and oligohydramnios (decreased amniotic fluid) (Gabow, 1998). Ultrasonography, magnetic resonance imaging (MRI) and computed topography (CT) are also used for diagnostic detection of PKD (Gabow, 1993). Diagnosis is commonly made based on the following criteria; patients younger than 30 years of age with the presence of at least two renal cysts unilaterally or bilaterally are considered a positive diagnosis. In patients 30 – 59 years if age, at least two renal cysts in each kidney is required and for the 60 plus age group, at least four

cysts in each kidney are required for a positive diagnosis (Ravine et al, 1994). The presence of extrarenal cysts, such as hepatic or pancreatic cysts can help ensure a proper diagnosis (Ravine et al, 1994).

2.2 Molecular and Cellular Pathophysiology

Although spontaneous mutation does occur, the frequency is relatively low, accounting for less than 10 % of all cases (Gabow, 1993). There are three known genotypes of ADPKD. The first, denoted PKD-1 is caused by a mutation on the short arm of chromosome 16. It is responsible for 85 to 90% of all ADPKD cases (Wunderle et al, 1994). PKD-1 is a large gene composed of 46 axons, spanning 53 kilobases of genomic DNA. It produces a 14.5 kilodalton (kd) mRNA that encodes a 4304 amino acid polypeptide, polycystin 1 (Van Adelsberg, 1999). It is thought to be involved in many biological processes such as carbohydrate recognition, ligand binding and calcium regulation (Murcia et al, 1998). It is also a protein that participates in cell-cell or cell-matrix interactions and regulates a signal transduction pathway mediated by specific protein-protein interactions at the cytoplasmic membrane (Avner et al, 1999).

Genetic studies in mice with targeted mutations show that polycystin-1 is essential for normal renal development and that its primary site of action is at the basal membrane adhesions of the ureteric bud epithelium (Wilson, 2001). Polycystin-1 is part of a complex that is necessary for the migration of embryonic renal cells. Activation of this gene seems to regulate other genes that are involved in regulation of proliferation and differentiation, both critical during development (Wilson, 2001). Therefore, a disruption in this polycystin-1 gene may alter cellular proliferation and differentiation and may alter

the migration processes in the embryonic ureteric bud, thus altering renal development (Wilson, 2001).

The second form, PKD-2 is a result of a mutation on the long arm of chromosome 4 and accounts for 5% to 10% of all ADPKD cases (Wunderle et al, 1994). The PKD-2 gene expresses a 4.5 kd mRNA that encodes a 968 amino acid polypeptide, polycystin 2 (Van Adelsberg, 1999). Polycystin 2 has a similar morphology to a voltage-activated calcium channel and it has been implied that it may be a subunit of a channel (Van Adelsberg, 1999). Current data suggests that polycystin 1 forms a heterodimer with polycystin 2 (Avner et al, 1999) and this may be the reason that both PKD-1 and PKD-2 have similar pathophysiology. A third form, PKD-3, has been identified but the location of this gene has not yet been determined (Grantham, 1995). This form of PKD has a very low occurrence of 1 to 5 % of all diagnosed ADPKD patients (Gabow, 1998).

There is only one known locus for ARPKD but there is great speculation that more exists due to the high variability in the progression and the severity of symptoms encountered (Gabow, 1998). The human gene for ARPKD has not yet been cloned but a mouse model for ARPKD, called *orpk* is providing insight into ARPKD (reviewed by Murcia et al, 1998). This model has a recessive mutation causing dual hepatorenal disease. In this model, the Tg737 gene was identified as the major mutant locus. Chromosomal mapping of this gene in humans has identified it be on chromosome 13 (reviewed by Murcia et al, 1998) and therefore cannot be the homologue to the gene involved in the primary form of ARPKD in humans, which is found on chromosome 6 (Gabow, 1998). However, the similarity in disease phenotypes makes it a compelling animal model for studying the molecular basis of renal cyst development. Recent

research has revealed that this gene has the potential to interact with polycystin 1 and other molecules that control epithelial cell polarity, epithelial growth factor receptor stability and cellular differentiation, all components that play a role in cyst development (Avner et al, 1999, Murcia et al, 1998).

2.3 Renal Function and Disease Progression

The kidneys have many functions and are vital organs necessary for good health. They are responsible for the excretion of wastes, the secretion of endocrine hormones and the regulation of body water, minerals and organic compounds (Briggs et al, 1998). Renal disease alters the ability of kidneys to function normally. An indication of renal disease is a reduction in glomerular filtration rate (GFR). Blood flows into the nephron via the afferent arteriole through the glomerulus to form an ultrafiltrate. The volume of fluid filtered from the glomerular capillaries per a unit of time is known as the GFR. A normal GFR is 65 - 130 ml/min/1.73 m^2 body area, generating 180 L of filtrate per day (as cited in Briggs et al, 1998). The GFR can be estimated by calculating creatinine clearance. Creatinine is a product of creatine, a protein found in muscles that is broken down at a constant rate. Creatinine is transported in the blood and excreted in the urine as a waste product. In renal disease, there is a reduction in GFR and the amount of creatinine that can be excreted is reduced causing urine levels to decrease and serum levels to increase. The following formula can be used to measure creatinine clearance:

creatinine clearance = urine creatinine x urine volume / serum creatinine

(as cited in Briggs et al, 1998)

This formula may however, overestimate creatinine clearance by approximately 10 % as the kidney produces creatinine and may slightly skew the estimated GFR.

In practice, measurement of creatinine clearance is useful in detecting renal disease and estimating the extent of renal impairment. A GFR between 25 and 70 ml/min/1.73 m^2 body area is considered mildly impaired renal function and once GFR falls below 20 – 25 % patients experience uremia, a build up of poisonous wastes in the blood causing nausea, vomiting, and fatigue. Once a patients reaches uremia and GFR falls below 10 ml/min/1.73 m^2 the patient is diagnosed with ESRD and will require dialysis or renal transplantation (Kopple, 1999).

The tubules of the kidneys are responsible for reabsorption of sodium, water, and other electrolytes and compounds. The absorption of sodium is normally about 99.5% and for water 99% of the total blood filtered (as cited in Briggs et al, 1998). Renal disease has a dramatic effect on renal tubule function. Early in the disease, inflammation, ischemia and scarring alter the tubules ability to reabsorb water, sodium and other compounds resulting in polyuria or the inability to concentrate water (Kopple, 1999). As the disease progresses, blood flow is reduced due to fibrosis and less blood can be filtered. Physiologically the reduction in blood flow causes the GFR to be slowed, causing an accumulation of fluid (resulting in anuria), sodium, potassium and phosphorus all to which are toxic to the body in higher or unbalanced quantities. Sodium and water retention causes edema while increasing potassium and phosphorus serum levels and increasing the risk of myocardial infarction and metabolic bone disease respectively. Diet modifications are implemented to minimize the amount of fluid accumulation and the

retention of sodium, potassium and phosphorus during early renal dysfunction but dialysis is necessary once patients reach ESRD (Kopple, 1999).

The kidneys are also responsible for the removal of metabolic wastes and foreign chemicals. Such things as urea, creatinine, uric acid and drugs are lethal if not removed from the blood circulation. These elements continue to build up in serum and cause the elevation of nitrogenous material causing azotemia and uremia. Once again diet can be manipulated to reduce the build up of nitrogenous wastes by reducing protein intake in early stages of renal insufficiency but again dialysis is used in later stages of renal insufficiency (Kopple, 1999).

The endocrine functions of the kidney are also affected during renal disease. The kidney secretes erythropoeitin, a glycoprotein that stimulates red blood corpuscle formation in the bone marrow. During renal failure, there is a decrease in the cells' ability to produce erythropoeitin leading to a reduction in red blood corpuscle production and anemia (Kopple, 1999).

The kidney plays an essential role in vitamin D metabolism as the kidney is responsible for converting vitamin D (25(OH) D_3, calcidiol) into its active form of 1,25 dihydroxyvitamin D_3 (1,25(OH)$_2D_3$, calcitriol). Calcitriol production is stimulated under low calcium conditions to increase serum calcium levels. When serum calcium levels drop, parathyroid hormone (PTH) is secreted and acts on the kidney to increase the conversion of calcidiol to calcitriol. Once calcitriol is produced, it increases reabsorption of calcium from the renal ultrafiltrate, liberates calcium from the bone matrix and increases dietary calcium absorption through the intestine (as cited in Delmez et al, 1998).

As the disease progresses the ability of the kidney to convert the inactive form of vitamin D to calcitriol is reduced. Serum calcium levels decrease because there is a reduction in the absorption of calcium due to lack of calcitriol and this causes a vicious cycle of low calcium levels and increasing PTH production that leads to secondary hyperparathyroidism. The high phosphorus level seen in renal disease is due to the inability of the kidney to excrete it. Phosphorus reduces the conversion of calcidiol to calcitriol and can also bind with calcium to form an insoluble phosphate crystal, both of which suppress calcium levels and increase PTH secretion (Ghazali et al, 1993).

The high levels of PTH cause an increase in the number of osteoclasts and promote resorption of the bone matrix to liberate calcium. This can be very detrimental in growing infants and children, as it can lead to improper formation of bone and destruction on the growth plate, leading to stunting and weak bones. However, in most cases, supplementation of vitamin D is effective at preventing renal bone disease and normalizing child bone growth (Maxwell, 1998). In adults, bone resorption causes areas of resorbed bone to be replaced with fibrous tissue that can not be mineralized due to a lack of calcium. This results in a reduction in bone quality and bones that are prone to fracture (Delmez et al, 1998).

Renin is another endocrine hormone that is secreted by the renal cells and is stimulated in response to altered glomerular blood flow. The juxtaglomerular cells of the renal afferent arteriole are sensitive to fluid volume and a decrease in blood flow causes these cells to secrete renin and activate the renin angiotensin aldosterone system (Granner, 1996). Renin acts on the substrate angiotensinogen to produce angiotensin I that is further converted to angiotensin II by the angiotensin converting enzyme (Granner,

1996). Angiotensin II increases renal blood pressure by causing vasoconstriction of the arteriole and is a potent stimulator of aldosterone. Together, aldosterone and angiotensin II increase fluid and sodium absorption in the kidney to increase the blood volume and blood flow rate (Granner, 1996). During early renal disease, cells undergo ischemia and renin is secreted to increase renal blood flow (Grantham, 1992). This increase in renal blood flow can causes further destruction and fibrosis and ultimately leads to a decrease in flow rate and causes an increase in renin secretion. This becomes a destructive cycle that often results in extreme hypertension and continual renal damage. Hypertension can greatly affect the progression of the disease and tends to be the diagnosing factor (Gabow, 1990).

Hypertension occurs in 50-75 % of subjects with ADPKD prior to the onset of renal insufficiency (Gabow et al, 1990). Hypertension occurs in 30 % of ADPKD children (Zeier et al, 1993), 60 % of adult patients prior to ESRD (Gabow et al, 1984) and 80 % of patients with ESRD (Milutinovic et al, 1984). In ARPKD, an estimated 60 % of ARPKD patients develop hypertension by the age of 15 (Gabow, 1998). The degree of hypertension relates to the severity of cyst involvement and renal damage.

Although hypertension is the most common complication seen in PKD, hyperlipidemia is also common in renal failure, occurring in 20 to 70% in patients experiencing ESRD (Jones and Kubow, 1999). Serum triglycerides and cholesterol do not circulate freely in the blood but are packaged in lipoproteins. An elevation of these lipoproteins and serum triglycerides is common in uremia due primarily to impaired catabolism of triglyceride-rich lipoproteins (Kopple, 1999). There is a reduction in the activity of plasma and hepatic lipoprotein lipase, the enzymes involved in lipoprotein

breakdown leading to impaired clearance of plasma triglycerides. Serum cholesterol also may increase due to a reduction in lecithin cholesterol acyltransferase and carnitine function may sometimes be impaired (Kopple, 1999).

2.4 Cyst Development

In ADPKD, cysts may arise from any part of the nephron (Lieske and Toback, 1993) but are most often found in the tip of the Loop of Henle's, the Bowmans space, and the proximal convoluted tubules (Goldman and Hartman, 1989). Renal cyst development in ARPKD differs from ADPKD as cyst development is confined to the collecting tubules of the nephron (Zak, 1997).

Early in the disease less than 5% of the nephrons are cystic with cysts varying in size from a few millimeters to a few centimeters and having a clear to cloudy coloured appearance, caused by tissue hemorrhaging (Welling and Grantham, 1991). In severe cystic kidneys, cysts are larger but there are relatively the same amount of cysts as less severe cystic kidneys suggesting that the progressive nature of this disease is not due to an increasing number of cysts but due to enlargement of the established cysts (Grantham et al, 1987). Of the 1 million nephrons found in each kidney, only 1 out of every 100 nephron tubule cells actually goes on to become cystic, the vast majority of nephrons are perfectly normal (O'Neill, 1997). Cystic kidneys can become severely enlarged and often reach weights of up to 8 kg, due mainly to fluid accumulation, and may exceed 40 cm in length (McCarthy and McMullen, 1997). A normal kidney has an average length of 14 cm and weighs an average of 0.30 to 0.35 kg (Forbes, 1999).

Research on cyst formation has focused on three areas of etiology and pathogenesis; epithelial proliferation, fluid accumulation and matrix remodeling. Cystic epithelia tend to have an irregular, polypoid shape and have an overall higher number of cells when compared to normal tubular epithelia (Grantham, 1987). The increase in epithelial proliferation has been attributed to abnormalities in the regulation of cell growth although no definite mechanism has been identified (Grantham, 1992). Renal cyst epithelia from human ADPKD kidneys grown in primary cell culture exhibit increased proliferative potential with increased numbers of cell divisions when compared to normal renal tubular epithelium (Wilson et al, 1992). Cultures of human polycystic kidney epithelia also have an increased response to growth factors such as epidermal growth factor (EGF) and a decreased response to inhibitors of proliferation such as tumor growth factor (Wilson, 1991a). A study by Klingel et al (1992) demonstrated that cyst epithelia have antigens associated with them that may cause a hyperproliferative state in undifferentiated tissues.

In order for a cyst to form, there must be intra-cavity accumulation of fluid in conjunction with the epithelial proliferation (Grantham et al, 1987). The accumulation of fluid in the cyst can be partially explained by regular glomerular filtrate flowing from the glomerulus into the tubule and into the forming cyst (Grantham, 1992). Once a cyst reaches several millimeters, it separates from the tubule and seals itself off. Cyst growth continues possibly by secretions from renal cells that surround the cyst (Grantham, 1992). Wilson and colleagues (1992) discovered a mislocation of the Na^{+}/K^{+}-ATPase pump from the normal basolateral to the apical surface which may contribute to the vectorial transport of solutes and water into the cyst lumen, causing the cyst to enlarge (Wilson et

al, 1991b). In early stages of cellular development of normal cells, the sodium pump is located on the apical surface but as the cell develops it is relocated to the basal surface (Briggs et al, 1998). Cystic epithelia lack normal brush border projections on the apical surface suggesting that cells do not fully differentiate (Grantham et al, 1987). It is unknown whether this mislocation of the sodium pump is due the lack of differentiation from an embryonic state or due to a signal or genetic mislocation (Woolf, 1998). However, the mislocation of the sodium pump cannot be essential for cyst growth since this finding has not been confirmed in human ARPKD kidney tissue (Avner & Sweeney, 1992).

Alterations have been noted in the tubule basement membrane and extracellular matrix of the cysts, causing an abnormal appearance of the basement membrane. Although immunostaining studies reveal that polycystic kidneys do contain all basement membrane constituents, primary culture reveals that cystic epithelium contains extracellular proteins that are not found in normal renal cells (Wilson et al, 1992). Very early in the disease, the basement membrane in the kidneys appears split and fragmented (Milutinovic and Agodoa, 1983). As the disease progresses, the basement membrane surrounding the renal cysts becomes an abnormal, thickened mass of interwoven fibrils (Wilson et al, 1992). The defects in the extracellular matrix have little uniformity in both humans and animal models (Gabow, 1993).

2.5 Extrarenal Manifestations

Similar cellular dysfunction and developmental alterations seen in renal tissue may be responsible for the development of extrarenal cystic manifestations. Hepatic cyst

formation is the most common extrarenal manifestation and occurs in approximately 50 % of all PKD patients (Gabow et al, 1990). There is an increased prevalence with increasing age, as it is rarely seen in children and often peaks between the ages of 50 and 60 (Gabow et al, 1998). Hepatic cysts are caused be increased numbers of dilated bile ductules and fibrosis in the portal areas (Patterson et al, 1982; Everson et al, 1990). The manifestations of hepatic disease include cholangitis and portal hypertension with esophageal varices and gastrointestinal bleeding (Gabow, 1998). Hepatic cysts only rarely affect hepatic function but can result in symptoms, most commonly chronic pain. In ADPKD, hepatic cystic disease occurs mainly in women and may be linked to female steroid hormones (Gabow, 1998). Oddly, children diagnosed with ARPKD earlier in life develop mild liver disease with significant kidney involvement, while those diagnosed later in life end up developing severe hepatic disease with mild renal destruction (Zak, 1999).

Cysts also manifest in other tissues of the body, such as the pancreas (10 %) and the spleen (5 %). Less commonly, cysts can be found in the thyroid, ovary, brain, pituitary gland, breasts, seminal vesicles, lung, parathyroid, pineal gland and peritoneum (Gabow, 1993). Intracranial aneurysms are common and occur in approximately 10-15 % of PKD patients (Goldman and Hartman, 1989). Other manifestations include; colonic diverticuli, found in 80 % of patients with ESRD (Gabow, 1990) and cardiovascular abnormalities, found in 26 % of PKD patients (Leier et al, 1984).

The exact mechanism of how extrarenal manifestations occur is still unknown. It is proposed by Gabow (1993) that the PKD gene directly or indirectly results in abnormal extracellular matrix and altered cell growth and may play a role in hernia formation,

diverticular disease and abnormal vascular tissue, which can lead to altered vascular reactivity and possibly intracranial aneurysms and cardiac-valve abnormalities. An altered vascular reactivity may play a role in hypertension, a common complication in PKD. The altered cell growth due to altered cell structure and mislocation of the Na^+/K^+ ATPase in conjunction with altered secretion results in cyst formation and growth.

2.6 Variation in disease progression

Although all individuals with PKD have a genetic mutation that results in the physiological development of multiple renal cysts, patients with PKD exhibit non-uniformity in the progression to ESRD. This variance has been attributed to numerous factors from gene location or form of PKD, gender differences, number of pregnancies, degree of hypertension and presence of hepatic cysts. Many of these are not modifiable making treatment and intervention of this disease a challenge.

The location of the defective gene plays a role in disease progression as PKD-2 tends to be a milder form of PKD and typically results in a later age of onset of renal insufficiency when compared to PKD-1 (Gabow, 1992). Hateboer and colleagues (1999) discovered that the median age of death or onset of ERSD was 53.0 years for individual with PKD-1 and 69.1 years for patients with PKD-2.

Both PKD-1 and PKD-2 demonstrate the same pathogenesis but PKD-1 progresses at a faster rate. Data suggests that the protein products of the PKD-1 and PKD-2, polycystin 1 and polycystin 2 interact and form a heterodimer structure at the cytoplasmic membrane (Van Adelsberg, 1999). It is suggested that PKD-1 has a much more severe outcome because polycystin 1 is structurally more important and may have a

higher functional activity in the heterodimer than polycystin 2. A mutation in polycystin 1 would therefore result in greater structural damage and less functional ability than a mutation in polycystin 2, resulting in a quicker progression to ESRD (Avner et al, 1998).

There appears to be a gender difference in the progression of this disease (Gabow, 1992, Gretz, 1989) although some studies have reported no gender difference (Simon, 1995) and some have reported a gender difference in only PKD-2 affected individuals (Hateboer et al, 1999). Generally males, both in human and animal studies, have an accelerated progression time or reach ESRD earlier than women (Gabow, 1992, Gretz, 1989, Cowley, 1993). On average, men have an earlier median age of ERSD (52.5 years) than women (58.0 years), a difference of approximately five years (Gretz et al, 1989). However, the gender effect seems to exert its influence in the earlier stages of the disease as both males and females at time of renal replacement therapy progress thereafter at the same rate (Gretz, 1989). A variety of explanations have been postulated for this gender difference, one being that expression of the PKD gene product is sex hormone dependent (Gretz et al, 1989). This is indicated by the differences seen between GFR in men and women. Men can have a reduction in GFR anytime throughout life, yet GFR in women starts to decline after menopause (Wesson, 1969). In addition, castration to male rats seems to attenuate the progression of glomerular injury in the renal ablation model (Levi et al, 1987).

Female reproductive hormones may also play a role in disease progression as women with hepatic cyst involvement and those women experiencing more than three pregnancies have an increased progression to ESRD (Gabow et al, 1992). In contrast, testosterone may also be a contributing factor as it has been shown to be renotropic in

females rats treated with testosterone, indicating that testosterone directly influences the progression of renal disease (Cowley et al, 1997). Testosterone may also influence the translation of the products of the renin angiotensin system in renal tissue and other systemic tissues (Gretz, 1989). As mentioned previously, this system increases blood volume by increasing resorption of sodium and creates a vicious cycle of increasing blood pressure and destruction to the renal tissue. This in turn may play a role in the progression of renal disease by causing hypertension (Gretz, 1989).

It has been suggested that cyst development increases at a slow but constant rate but a secondary infection or development of hypertension contributes to the rapid decline to end stage renal failure (Franz and Reubi, 1983). Early renal disease plays a role in the development of hypertension and hypertension plays a role in the progression of renal disease (Gabow, 1992). Gabow and colleagues (1990) demonstrate a relationship between structural deformation and hypertension early in the course of ADPKD. Hypertension was associated with greater renal structural abnormalities and renal volume at comparable body surface areas and renal function. These authors suggested that the early initiation of hypertension in ADPKD might be due to renal ischemia secondary to cyst alteration of renal blood flow, presumably due to activation of the renin-angiotensin-aldosterone system.

Although there are a variety of internal factors that alter the severity and progression of PKD, there are environmental factors such as medications and dietary modifications that have been shown to modify the progression of renal disease. Current drug therapies are used to treat secondary disorders associated with PKD to decrease the rate of destruction in the kidney and slow the progression to ESRD. For example, methyl-

prednisolone and angiotensin converting enzyme inhibitors are used to decrease inflammation and hypertension respectively. Currently the role of diet is under investigation to determine its preventative and therapeutic role in chronic renal disease.

2.7 Protein and Renal Disease Progression

The role of dietary intervention on renal disease progression, especially the role of protein, is the subject of current research. An association between protein and renal disease was first made in 1836 by Richard Bright when he suggested that individuals suffering from renal insufficiency should restrict protein intake to lower the kidney work load (as cited by Velasquez and Bhathena, 2001). Research has confirmed that high protein intake has a detrimental effect on renal structure and accelerates the progression of renal disease (Brenner et al, 1982; Williams et al, 1987; Tomobe et al, 1994; Ogborn et al, 1995). Initial studies examining the role of protein on the kidney discovered that excessive protein intake causes hyperfilatration, and glomerular hypertension and a progressive deterioration of kidney function in the renal ablation model of chronic renal disease (Brenner et al, 1982).

Subsequent research established that low protein diets in healthy humans is associated with lower renal plasma flow, lower GFR and less albumin excretion than those seen in normal protein diets (Viberti et al. 1987). Results have been replicated in a variety of animal models. In the *pcy* mouse model, low protein diets (6 % vs 25% of protein by weight of casein) resulted in less renal enlargement, and a 46 % lower total cyst area in mice fed the 6 % compared to the 25 % protein diet (Aukema et al, 1992). In the Han:SPRD-*cy* rat model, low protein intake (8 % vs 20 % by weight of casein

protein) resulted in increased survival time and a reduction in total cyst volume when compared to nonaffected animals. Mean serum creatinine and urea levels were significantly lower in the low protein fed animals (188 mmol/L and 15.6 mmol/L) when compared to the 20% protein fed animals (272 mmol/L and 81.5 mmol/L) (Ogborn et al, 1995).

Although most data in human trials are inconclusive, some human studies have produced positive results. Maschio and colleagues (1983) examined the effect of low protein (0.6 g of protein per kg body weight) on renal patients ranging from 15 to 68 years of age. This study demonstrated that dietary protein restriction was effective at delaying progression in most patients with early to moderate renal failure, but had little effect on patients with severe renal failure. Similarly, Alverstand and colleagues (1983) reported protein restriction of 15 to 20 g/day in middle aged patients with renal insufficiency lowered serum creatinine levels. Preliminary data extracted from Chapman and colleagues demonstrated that individuals with PKD consuming a protein diet equivalent to the recommended daily intake (0.8 g protein/kg/day) had smaller or less cystic kidneys than those patients with normal protein intake (reviewed in O'Neill, 1999).

A large clinical trial was conducted by the Modification of Diet in Renal Disease Study Group to determine if protein restriction influenced renal disease progression. This study demonstrated a slight benefit of protein restriction (0.58 g/kg/day) over the typical protein intake (1.3 g/kg/day) in patients suffering from moderate renal insufficiency. However, no benefit of either lower or very low protein (0.28 g/kg/day) was detected in those patients with severe renal disease (Klahr et al, 1994). Secondary analysis conducted

using the same data set has demonstrated a more conclusive beneficial effect of protein restriction over the original study (Levey et al, 1999).

There are a number of reasons why animal models have greater success at altering renal disease progression than human studies. Firstly, these discrepancies may be due to the actual amount of protein intake. In humans, studies examining the role of low protein intake or protein restriction on disease progression often prescribe the recommended protein intake of 0.8 g/kg/day, while the high protein diet is more the typical protein intake of 1.3 g/kg/day. However, the problem with human studies is the lack of control over dietary intake of protein and the lack of compliance of the participants. Many studies demonstrate that patients often consume more protein than prescribed by the study protocol, making it difficult to examine the true role of protein on disease progression. Lack of compliance is not a problem in animal studies as all animals consume identical diets that contain equal amounts of protein. Therefore, because it is easier to control dietary intake in animal models, the data from these studies provide more consistent and dramatic results.

The length of the feeding trial differs between human and animal models in respect to total life span. A human study conducted over a 2-3 year period is considered a long trial, yet when examining the influence of a 2 –3 year dietary intervention over a lifetime, the intervention time is relatively short. In animal studies, a shorter trial of 8 weeks reflects a longer intervention time as animal models may have a much shorter life span. An increase in dietary intervention with respect to duration over the life span would increase the chance that protein restriction would exhibit a beneficial affect of attenuating renal disease.

2.8 Soy and Renal Disease

In 1987, Williams and colleagues experimented with protein sources as well as different protein amounts using the renal ablation model. The renal ablation model is an accepted and widely used model of chronic renal disease. Rats were subjected to either a 12 or 24 % soy protein diet or a 12 or 24 % casein diet for 12 weeks. Soy protein demonstrated beneficial effects on the renal ablated kidney, irrespective of the amount of protein (12 % or 24 % protein intake). Soy fed rats demonstrated less proteinuria (35.5 vs 63.8 mg/24 h), less hypertrophy, less pathological lesions of glomerular sclerosis (42 % vs 67 %) and tubular atrophy (0.60 ± 0.16 vs 1.57 ± 0.20) with a lower mortality rate (95 % vs 61 %) than casein fed animals. This study concluded that the quality of protein also affects renal disease progression and that vegetable protein has a less detrimental effect on renal tissue than animal protein.

Recently, research investigating the role of soy in renal disease and in other chronic diseases has increased dramatically. In the area of chronic renal disease, a soy based diet seems to attenuate the progression of PKD (Ogborn et al 1998, Aukema et al, 1999; Aukema et al, 2001; Tomobe et al, 1998). Soy protein feeding in male Han:SPRD-*cy* rats for 6 weeks reduced the number of renal cysts (0.98 vs 4.92 ml/kg body wt), fibrosis (0.79 vs 1.4 ml/kg), macrophage infiltration, renal tubular cell proliferation and apoptosis when compared to a casein based diet (Ogborn et al, 1998). Similar results were found in the *pcy* mouse model of PKD after 13 weeks of feeding soy protein. Animals had a 28% lower kidney weight, a 37% reduction in cyst scores (% cyst area x relative kidney weight) and 25% less kidney water, all indicating a slower progression to ESRD (Aukema et al, 1999).

The exact mechanism of how soy attenuates chronic renal diseases such as PKD is unknown. Soybeans have a high protein quality and content, containing 35 % protein, which is much more than other legumes (Messina, 1995). Soy protein has been recognized as having potential roles in the prevention and treatment of many chronic diseases, from heart disease to cancer (Messina, 1995).

A number of components in soy may be responsible for its beneficial effects in health and disease states. Soybeans contain genistein, a phytoestrogenic isoflavonoid that has been shown to influence sex hormone metabolism and biological activity as well as influence intracellular enzymes, protein synthesis, growth factors, malignant cell proliferation and angiogenesis (Aldercreutz et al, 1995).

Genistein is an inhibitor of tyrosine protein kinase activity (Aldercreutz et al, 1995). Tyrosine kinases are involved in cell proliferation and oxidative stress (Lan et al, 1994). Cyst development is caused in part by an increase in tubular cell proliferation and it is suggested that the decrease in cyst development and disease progression seen in soy feeding may be due in part to the inhibition of tyrosine kinases (Grantham, 1996; Cowley et al, 1993). Soy may also have anti-inflammatory effects since tyrosine kinase activity is associated with cytokine and growth factor activation and proliferation of inflammation mediators (Velasquez and Bhathena, 2001). However, most studies on tyrosine kinase inhibition and genistein are in vitro studies that use genistein levels that are not achievable through dietary consumption of isoflavonoids. This may be why genistein supplementation to a casein diet did not reduce cyst and kidney size in the *pcy* mouse (Tomobe et al, 1998) and suggests that genistein may not be the major contributing factor of how soy attenuates renal disease (Tomobe et al, 1998; Ogborn et al, unpublished).

Soy protein feeding has also been shown to reduce liver Δ6 desaturase activity, an enzyme in PUFA metabolism (Lindholm and Eklund,1991). Although the exact mechanism of how soy alters Δ6-desaturase activity is unknown, it is known that its activity is influenced by dietary and hormonal factors. For example, diets low in essential fatty acid, high in protein or low in fat increase Δ6-desaturase activity while low protein intake, alcohol, and secretion of epinephrine, glucocorticoids and glucagon decrease its activity (Jones and Kubow, 1999).

Soy may alter Δ6-desaturase activity through its slightly different amino acid composition. Soy protein has a higher arginine content, 7.5 % while in casein protein it is 3.7 % of the total amino acids (Huang et al, 1986). For a complete list of the amino acid composition of both proteins, refer to Table 1. Arginine is a precursor for glucagon and stimulates glucagon secretion upon ingestion (Granner, 1996). One of the biological functions of glucagon is to reduce Δ6-desaturase activity to increase glucose levels. Therefore, it has been postulated that the higher arginine content in soy may increase glucagon levels and thereby inhibit Δ6-desaturase activity (Huang et al, 1986).

Soy protein also demonstrates an affect on fatty acid metabolism, which may be linked to the reduction in Δ6-desaturase activity. Ogborn and colleagues (2000) discovered that soy fed Han:SPRD-*cy* rats had an enrichment of renal and hepatic linoleic acid and a lower proportion of hepatic arachidonic acid (AA), implying alterations in PUFA metabolism. These alterations in fatty acid status could reflect a modification in eicosanoid production.

Table 1 Amino acid composition of casein and soy protein (Harlan Teklad, Madison, Wisconsin)

Amino Acid	Casein (%)	Soy (%)
Alanine	3.0	4.1
Arginine	3.7	7.5
Aspartic Acid	6.9	11.9
Cystine/Cysteine	0.4	1.3
Glutamic Acid	20.9	21.5
Glycine	1.8	4.2
Histidine	2.9	2.6
Isoleucine	4.6	4.9
Leucine	9.1	8.1
Lysine	7.7	6.3
Methionine	2.9	1.3
Phenylalanine	5.1	5.4
Proline	10.4	5.5
Serine	5.8	5.2
Threonine	4.3	3.7
Tryptophan	1.2	1.5
Tyrosine	5.5	4.0
Valine	5.7	4.5

amino acid composition does not equal 100 for either protein due to variability in amino acid analyses. These values indicate the most typical values from the producers database.

2.9 Eicosanoid Production

Eicosanoids are oxygenated unsaturated 20 carbon fatty acids that include PGs, thromboxanes, prostacyclin and leukotrienes that are further divided into series depending on their precursor. Figure 1 demonstrates the n-6 pathway where linoleic acid (LA) is catalyzed by Δ6-desaturase to form γ-linolenic acid (18:3n-6) and further elongated to dihomo- γ-linolenic acid (20:3n-6), a precursor to the 1 series of eicosanoids. Dihomo- γ-linolenic acid is desaturated by Δ5-desaturase to produce AA (20:4n-6), a precursor to the 2 series of eicosanoids. In the n-3 pathway (Figure 2), α-linolenic acid (LNA) is converted to eicosapentanoic acid (EPA) via desaturation and elongation and is the precursor to the final series of eicosanoids, the 3 series. The 2 series of eicosanoids are more potent vasoactive compounds than the 1 and 3 series of eicosanoids (Jones and Kubow, 1999).

In unstimulated cells AA is esterfied with acetyl CoA and is then transferred into the 2-acyl position of phospholipids in the cell membrane. In stimulated cells AA is liberated by the hydrolysis of membrane phospholipids, phosphatidylethanolamine, phosphatidycholine and phosphatidylinositol by the enzyme phospholipase A_2 (PLA_2).

Once liberated, AA can be further elongated and desaturated or can enter the eicosanoid pathway as seen in Figure 1 (Jones and Kubow, 1999). In the cyclooxygenase pathway, AA is catalyzed by two highly homogenous isoforms of the cyclooxygenase (COX) enzymes to produce prostaglandin endoperoxides which will subsequently become either prostaglandins or thromboxanes (Mayes, 1996). These two isoforms are

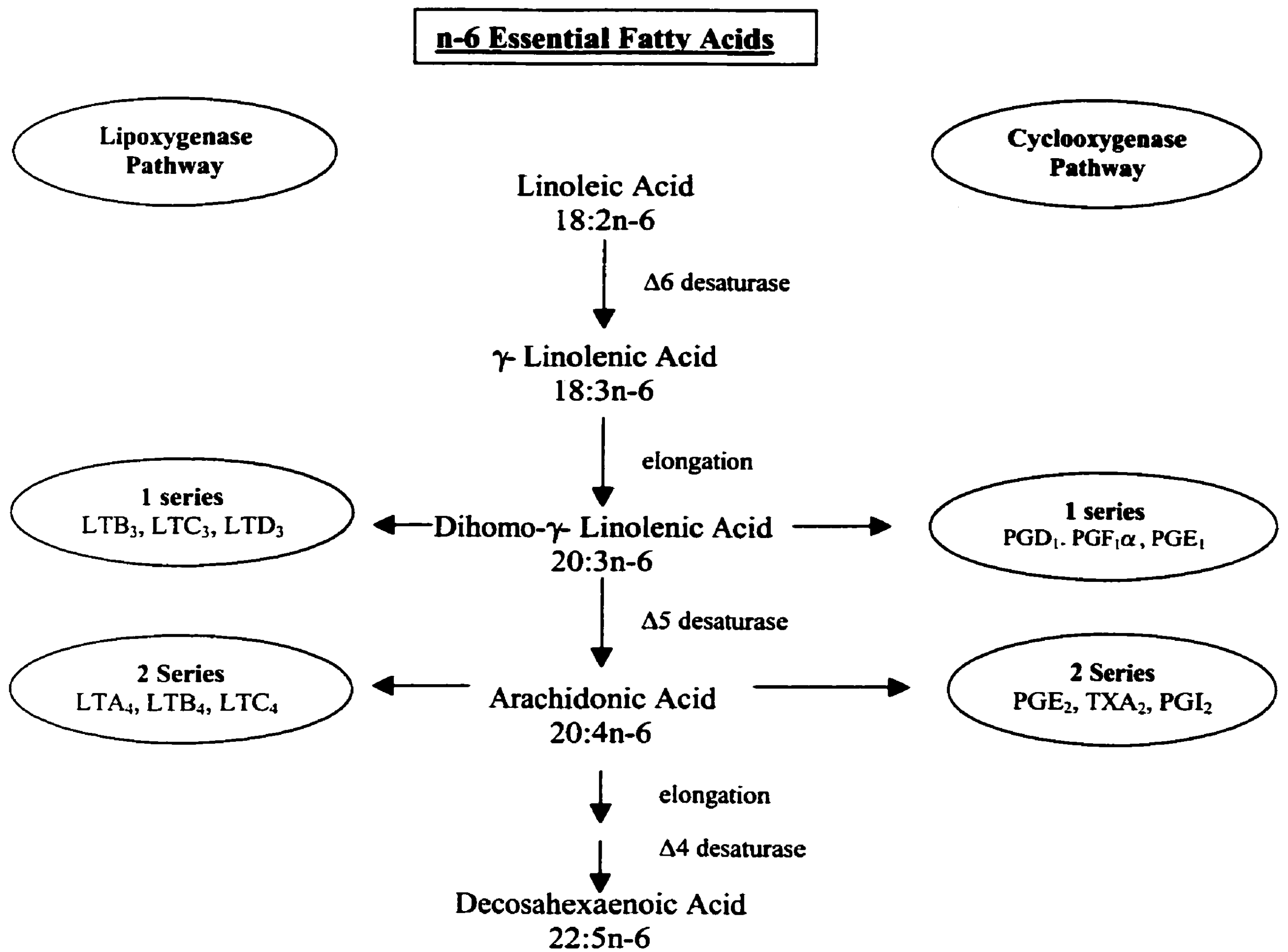

Figure 1 The n-6 essential fatty acid metabolism pathway and corresponding series 1 and 2 eicosanoid production. (modified from Jones and Kubow, 1999)

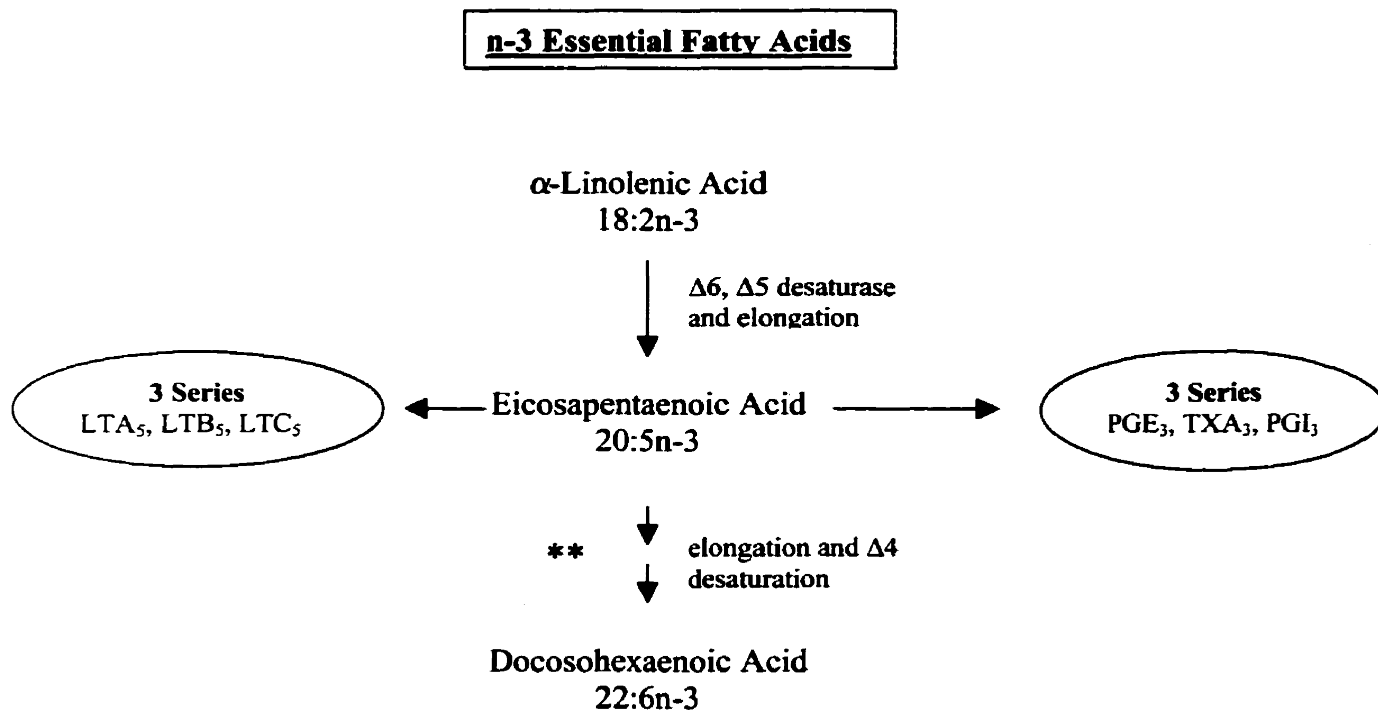

Figure 2 The n-3 essential fatty acid metabolism pathway and corresponding series 3 eicosanoid production. (modified from Jones and Kubow, 1999).

** Sprecker et al (1989, 1995) has speculated an alternate pathway from EPA to DHA involving elongation of EPA followed by Δ6 desaturation in the microsomes followed by β-oxidation to produce DHA.

known as COX-1 and COX-2 and are similar in amino acid sequence and enzymatic function but have slightly different physiological functions (Sanchez et al, 1999). COX-1 is present in most tissues but at different levels in various cell types while COX-2 is usually undetectable in most tissues but can be inducted under certain conditions, such as ischemia (Sanchez et al, 1999). However, in the kidney, COX-2 can be found under basal conditions.

PGs are produced in a variety of tissues, including the kidney although their role in normal renal physiology is not yet fully understood. In renal tissue, PG synthesis proceeds principally via the cyclooxygenase pathway leading to the production of PGs and thromboxanes. Many regions of the kidney are capable of producing PGs and the exact PG formed varies from region to region and may reflect the functional requirements of that particular region. For instance, PGI_2 is a renal prostaglandin that is found in large amounts in the epithelium of the afferent arteriole and acts as a vasodilator (Williams and Peck, 1977). $PGF_{2\alpha}$ is found in the glomerulus and PGE_2 is found principally in the medulla and is a vasodilator (Williams and Peck, 1977), a mediator of inflammation (Trang, 1980), a stimulator of cell proliferation (Horrobin, 1978) and a chemotactic agent on leukocytes (Higgs et al, 1975).

The vasodilatory eicosanoids PGE_2 and prostacyclin and the vasoconstrictors angiotensin II and TxA_2 synergistically regulate GFR and renal plasma flow (Schor et al, 1981). PGE_2 plays an important role in the autoregulation of GFR during sodium and water depletion by increasing renal sodium excretion through renal vasodilation and direct inhibition of tubular sodium reabsorption. PGE_2 also increases renin release by a direct effect on the juxtaglomerular cells, and is thus indirectly responsible for

angiotensin II and aldosterone production. Angiotensin II promotes sodium retention and sodium reabsorption, is a potent renal vasoconstrictor, and lowers GFR while aldosterone increases sodium reabsorption and potassium excretion. PGE_2 also facilitates water excretion by decreasing sodium reabsorption in the loop of Henle and by inhibiting the hydro-osmotic effect of vasopressin (Krishna et al, 1988).

Disease and eicosanoid production are closely linked. The overproduction of AA eicosanoids has been implicated in many chronic inflammatory diseases (Peck, 1997; Sanchez et al, 1999). Animal models of chronic renal disease have been shown to alter COX enzyme activity and eicosanoid production. Sanchez et al (1999), demonstrated an increase in COX-2 mRNA and increased COX-2 enzymatic activity in renal ablation, suggesting an induction of COX-2 mRNA expression and production during renal disease. Aukema and Jiang (2000) reported a lower level of COX-2 enzyme in diseased Han:SPRD-*cy* rats and *pcy* mice, both models exhibited an increase in both COX-1 and PLA_2 enzymes. These alterations in COX and PLA_2 enzymes may lead to modifications in eicosanoid production by increasing the level of vasodilatory PGs such as PGE_2, that may participate in renal tissue destruction and accelerate the progression of renal disease (Sanchez et al, 1999).

In addition to disease, protein also alters eicosanoid production. Paller and colleagues, (1986) revealed that animals receiving a high protein diet had an increase in urinary PGE_2 excretion. Yanagisawa and Wada, (1998) concluded that high protein intake resulted in higher levels of PGE_2, COX and PLA_2 due to selective enhancement of eicosanoid production via activation of the PLA_2-COX pathway. Kontessis and coworkers (1990) went further to examine the types of protein and discovered that meat

protein produces an increase in the vasodilatory prostaglandins (PGE_2, 6 keto $PGF_{1\alpha}$ and TxB_2) but soy protein had no affect on the production of prostaglandins. The increase in the vasodilatory prostaglandins, PGE_2, $PGF_{1\alpha}$ and TxB_2 was suggested to be mediated by an increase in PLA_2 activity and an increase in arachidonate release (Stahl et al, 1987). Although this study does not examine these enzymes, it does examine the vasodilatory PGE_2 to determine if soy protein does alter its production and if soy does alter PGE_2, further investigation on these enzymes will be warranted.

2.10 Early Dietary Intervention

Although PKD manifests itself during the fifth to sixth decade of life in humans, this disease is an infantile disease as cyst development and alterations in renal tissue begin early in life. It is not surprising that studies have shown that dietary intervention has little effect on later stages of renal sufficiency (Locatelli et al, 1991, Maschio et al, 1983), as cysts are fully developed and much of the renal tissue is destroyed. This data stresses the importance of early dietary intervention and to modify cyst development when it starts, early in life.

As previously discussed, soy protein has been shown repeatedly to reduce cyst volume, serum creatinine and slow disease progression (Ogborn et al, 1998; Aukema et al, 1999; Ogborn et al, 2000; Aukema et al, 2001). Although some researchers have begun dietary intervention post weaning, studies examined longer term soy feeding making it impossible to predict how soy influences the early development of the disease.

A short term early dietary intervention on the other hand could demonstrate the early influences of soy on renal function and disease progression prior to or during early

renal modification such as cyst development. A short term trial would also demonstrate whether short term feeding can influence disease progression or whether long term feeding of soy protein is necessary.

Certain considerations must be taken into account, however, when dealing with post weaning animals. These animals are still undergoing growth and development and may require more energy for growth and development and require specific fatty acids for development of certain tissues. For example, LA and LNA are essential fatty acids (EFA) and their derivatives AA and DHA are essential for optimal growth and for visual and neural development (Uauy and Hoffman, 2000; Crawford et al, 1992; Koletzko and Braun, 1991; Innis, 1991; Salem and Niebylski, 1993). The increased requirement for these fatty acids may alter the renal and hepatic fatty acid status of these components.

On top of the different requirements for growing animals, renal disease may also alter fatty acid composition by modifying the ability of the tissues to produce or utilize the adequate supply of these nutrients. Disease may interfere with fatty acid metabolism, as seen in glomerulonephritis animal models that had lower renal LA and EPA levels (Das et al, 1993). The disease state may increase the release of AA from membrane phospholipids for production of eicosanoids, as PGs have been implicated in chronic renal injury. An increase in PG formation in renal disease may deplete AA and EPA pools for its production leaving little for development, although few studies have examined renal disease and the alterations in nutrient utilization.

2.11 Han:SPRD-*cy* Rat Model

This research was conducted using the Han:SPRD-*cy* rat model of PKD. This model arose spontaneously in the Sprague-Dawley rat in 1986 (Kaspareit-Rittinghausen et al, 1991) and has been identified as a key rodent model for human PKD. This rat model carries an autosomal dominant trait with the PKD gene located on chromosome 8 (Kaspareit-Rittinghausen et al, 1991). One quarter of the animals born to heterozygous parents, will be homozygous rats and will develop severe renal disease that is fatal within the first 3 to 4 weeks of (Gabow, 1990). The heterozygous animals, accounting for two thirds of the remaining animals become affected while one third do not acquire a defective gene and are normal. In heterozygous rats, renal abnormalities closely resemble those seen in humans (Cowley et al, 1996). Firstly, cyst development begins perinatally or earlier *in utero* and cysts are located in any segment of the nephron. The heterozygous male also develops interstitial fibrosis that is associated with progressive deterioration of renal function.

The time course of development is similar to humans on a relative scale with manifestation before sexual maturation with renal failure occurring mid-life (Cowley et al, 1996). The Han:SPRD-*cy* model also shares the gender difference seen in humans, however, female rats progress at a strikingly slower rate than in human females. Heterozygous males resemble human PKD development with symptoms of chronic progressive azotemia and demonstrating similar structural abnormalities in the renal basement membrane and in cellular proliferation (Cowley et al, 1993). This model also shares similar manifestations seen in humans such as renal osteodystrophy, uremia and renal hypertension and development of hepatic cysts (Cowley et al, 1996). These

similarities allow researchers to study the relationships between structural renal alterations and the extrarenal symptoms of chronic renal disease (Kaspareit-Rittinghausen et al, 1990).

Another PKD model is the murine *pcy* mouse, which arose spontaneously in the diabetic KK mouse strain (as cited in Aziz, 1995). The murine PKD gene is located on mouse chromosome 9 with the disease being inherited in an autosomal recessive fashion trait rather than a dominant one as seen in humans and the rat model. This model differs from the rat model as the disease progression is slower in the mouse model (Aziz, 1995).

However, for this particular thesis research, the close resemblance to humans and the quick disease progression makes the male Han:SPRD-*cy* rat model a good model for examining early dietary intervention.

3 Methods and Materials

Experimental Design

Male, Han:SPRD-cy rats (n=87) were provided by the breeding colony of Dr. M. Ogborn (University of Manitoba, Winnipeg, MB) that originated from breeding stock provided by Dr. Benjamin Cowley (University of Kansas Medical Center, Kansas City, KS). The experimental design was examined and approved by the University of Manitoba Committee on Animal Use and is in agreement with the guidelines of the Canadian Council on Animal Care.

Rats were randomly divided into two groups and placed on either an experimental or a control diet at weaning (3 weeks of age). The two diets were identical with the exception of the protein source. Both diets were manufactured in the lab and were based on the AIN-76 rodent diet (Bieri, 1979) but contained the vitamin and mineral mixes of the AIN-93 diet (Reeves et al, 1993). A complete composition of both diets is listed in Table 2. Control animals were fed a diet containing 20% by weight of casein protein and the experimental group was placed on a 20% by weight soy protein based diet. Animals fed soy protein had unlimited access to food. Each day food intake was measured and the amount of intake for soy fed animals was established and this amount was given to the casein fed animals. Animals were housed in groups of 2 to 5 animals per cage with the animal facility maintained at 22 to 24 ° C with 50 to 67 % relative humidity and a 12 hour light/dark cycle.

One day prior to termination, rats were placed in metabolic cages for a period of 6 hours to obtain urine samples beginning at 7am. During this time, food and water were withheld to prevent sample contamination. Mineral oil was used in the urine collection to

Table 2 Composition of control and experimental diets. Diet was in reference to the AIN-76 purified diet for rodents (Bieri, 1979) with modified vitamin and mineral mixes (Reeves et al, 1993).

Component	Content % by weight
Protein	20 casein or soy
d-l methionine	0.3
Corn Starch [a]	52
Dextrose [b]	13
Cellufil	5
Mineral Mix – AIN-93	3.5
Vitamin Mix – AIN-93	1.0
Choline bitartrate	0.2
Mazola Corn Oil [c]	5
* Total fatty acid composition (% total lipid)	
16:0 palmitic acid	9.9
18:0 stearic acid	1.7
18:1 (cis-9) oleic acid	26.1
18:1 (cis-11) vaccenic acid	0.5
18:2ω6 linoleic acid	60.4
18:3ω3 α-linoleic acid	1.0
20:0 arachidic acid	0.4

all components with no symbol-supplied by Harlan Teklad, Madison, Wisconsin.
[a] -supplied by the University of Manitoba, Winnipeg, MB
[b] -supplied by The Brew Doctor, Winnipeg, MB
[c] -supplied by The Real Canadian Superstore, Winnipeg, MB
* fatty acid composition of corn oil

prevent evaporation. Once samples were collected the mineral oil was removed and samples were weighed to determine total weight of the urine. The samples were then frozen at –20°C for later analysis.

Animals were terminated by exsanguination under sodium pentobarbital ("Somnotol" MTC Pharmaceuticals, Hamilton, On) anesthesia (65mg/kg, intraperitoneally). Once anesthetized, animal weights were recorded and blood was drawn via heart puncture for blood plasma and serum samples. Approximately 5 to 8 ml of blood was drawn and then separated into either a heparin-coated vacutainer for plasma or a sterile vacutainer for serum. Samples were centrifuged using a Beckman Avanti J-25 I Centrifuge (Mississauga, Ontario) for 10 minutes at 739 *g* for separation and plasma/serum was removed from the vacutainer, placed into cryogenic tubes and stored in a –20°C freezer for later analysis. Serum creatinine, triglycerides and cholesterol were measured using serum samples.

After exsanguination of the animal, the abdomen was opened, both kidneys were excised, weighed and then sectioned into consistent pieces. The left kidney was divided vertically and horizontally to provide four equal sections, while the right kidney was sectioned into thirds. The center section of the right kidney was placed in formalin for histopathology, and the bottom third was place in 10% Hanks Salt Solution (Sigma Pharmaceuticals, H-4385) for PGE_2 analysis. All other sections were placed in cryogenic vials, snap frozen in liquid nitrogen and stored at –80 °C until analysis. Liver tissue sections were also obtained and snap frozen and then stored for lipid analysis.

3.1 Histopathology

3.1.1 Disease Diagnosis

Diagnosis of diseased and normal animals was made post mortem using an Olympus BX60 microscope (Olympus Optical Co, Japan). High power magnification was used to ensure that lymphatic or empty vascular spaces were not mistakenly counted. Animals were classified as polycystic if examination of a single longitudinal cross-section of renal tissue contained over 10 areas of tubular dilation with an increase in extracellular matrix.

3.1.2 Renal Cyst and Fibrosis Volume

A section from the right kidney was used for histological analysis. Slices of renal tissue were place in 10% formalin for 120 minutes then embedded in paraffin wax and sectioned at 5 microns using a Microtome (American Optical 820, Southbridge, Massachusetts). For determination of renal cyst volume, sections were stained with hematoxylin and eosin as this stain illuminates cellular wall and nuclear proteins allowing for cell identification (complete detail of staining protocol can be found in the Appendix section). Quantitative analysis on renal fibrosis was conducted using aniline blue stain only in adaptation of Masson's trichome stain (Cohen, 1975). It has been demonstrated that aniline blue staining alone radiates collagen type III (a component of fibrosis) with the use of a standard incandescent microscope light source.

3.1.3 Image analysis

Image analysis was performed using a system that contained a Spot Junior CCD camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) mounted on an Olympus BX60 microscope with images being captured using Spot software Version 3.0.1 (Diagnostic Instruments Inc) and image analysis being conducted using the Image Pro Plus Version 4.10 software package (Media Cybernetics, Del Mar, California).

Renal cyst volume was determined using the Cavalieri principle (as cited in Ogborn et al, 1995). Renal tissue was sectioned into 2.5mm strips using a Lucite guide box. A video image was taken of each section with a linear scale using module 2500 of the Imagemeasure software package (Phoenix Biotechnology, Seattle, Washington, USA) to determine renal volume as calculated as the product of total section area and section thickness.

Using a 2x objective, a 64 x 64 pixel rectangle was moved in an alternating horizontal and vertical path until 25 measurements were obtained from each of four separate kidney sections. An automated measurement sub-routine was used to determine the areas of tubular lumen or cyst areas by hue and intensity characteristics. Renal volume was expressed as a ratio of the area identified as tubular lumen or cyst to the total image area, excluding any areas beyond the renal capsule. A measurement was accepted if the 95% confidence intervals of the mean were within 2% of the mean. Renal fibrosis volume was measured in a similar way, with the end product being a result of the ratio between areas that had taken up aniline blue stain to total image area. The product of the proportion and the reference renal volume corrected for body weight gives the final volume occupied by renal cyst volume or renal fibrosis volume.

3.2 Chemistry

3.2.1 Urine Creatinine Analysis

Urine creatinine was measured using the Sigma Creatinine Kit 555-A. Standards were prepared to test linearity using 15 mg/dL of creatinine standard (Sigma 925-15) and controls were prepared using 3mg/dL creatinine standard (Sigma 925-3). The 96 well microplate was labeled for identification purposes. The reagent was prepared using alkaline picrate reagent in a 5:1 ratio of alkaline picrate:sodium hydroxide supplied in the Creatinine Kit. Each sample was diluted 20 fold and placed in duplicate into the wells using a volume of 20 μl per well. 200 μl of alkaline picrate reagent was added to each well and was incubated for 10 minutes at room temperature. During this time, the reagent and creatinine bind to form a yellow/orange colour. The more creatinine in the sample, the deeper or stronger the colour becomes. After incubation the colour intensity was measured at 500 nm in a Powerwave X spectrophotometer (Biotek Instruments Inc,Winooksi, Vermont) using Kineticalc-KC4 software – version 2.5 rev 17 (Biotek Instruments Inc, Winooksi, Vermont). Values were multiplied by 20 (to correct for the dilution) and then multiplied by 88.4 to convert the value to μmol/L.

3.2.2 Serum Creatinine

The same procedure was completed for the serum creatinine analysis with the exception that plasma samples were not diluted. The above procedure was carried out, but, once the first creatinine concentration was calculated, further steps are required for determination of serum creatinine as serum contains many other proteins than urine that

can bind with the colour reagent and would give a false reading. Once the initial reading at 500nm was complete, the plate was removed from the spectrophotometer and 6.6 μl of acid reagent (a combination of sulfuric acid and acetic acid, reference # 555-2) was added to each well. The plate was sealed with Costar plate sealer and was placed on a Cooke Laboratory Micro-shaker (Division of Dynatech Laboratories Inc., England) for 5 minutes to remove precipitate. The acids cause the creatinine-picrate colour to fade faster than the interfering protein-picrate chromogens. The plate was then read a second time at 500 nm to determine the colour intensity. The difference found in colour intensity before and after acidification is proportional to creatinine concentration. Creatinine concentration was calculated using the following equation:

$$\text{Creatinine Concentration} = [(\text{initial}_{A\ \text{unknown}} - \text{Final}_{A\ \text{unknown}}) / (\text{initial}_{A\ \text{std}} - \text{Final}_{A\ \text{std}})] * 88.4\ \mu\text{mol/L}$$

3.2.3 Creatinine Clearance Calculation

Creatinine clearance is an indicator of renal function and correlates with GFR. In this experiment, creatinine clearance was used as a measure of renal function and disease progression as a low clearance level is a sign of deteriorating kidney function. Once serum and urine creatinine levels were obtained using a creatinine kit, creatinine clearance was measured using the following formula:

Creatinine Clearance = urine creatinine x urine volume / serum creatinine

Note: urine creatinine, serum creatinine and urine values for each group can be found in the Appendix.

3.2.4 Triglycerides

Serum triglyceride levels were measured using a Sigma Infinity Triglyceride Kit (344-20). Standards and controls were prepared to obtain a linear relation line with the standard (Accuset Liquid Calibrator A2539, Sigma Diagnostics) and control (Glycerol Standard, Sigma G-1394).

Samples were diluted by mixing 15 μl of sample serum with 45 μl of saline and 10 μl of diluted samples, standards and controls were placed in duplicate into each well. 200 μl of reagent was added to each well and the plate was incubated at 37°C for 5 minutes. During incubation a number of steps take place. Triglycerides are hydrolyzed by lipoprotein lipase to give glycerol and free fatty acids. The glycerol is phosphorylated in the presence of ATP and glycerolkinase to form glycerol-1-phosphate and ADP. The glycerol-1-phosphate is oxidized by glycerophosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide and 4-aminoantipyrine and N-ethyl-N(3-sulphopropyl)m-anisidine are catalyzed by peroxidase to form a quinoneimine dye that is quantified at 540 nm. The more triglyceride in the sample the more dye will be formed and the more intense the colour will be. Once incubation was completed, the absorbance was read at 540 nm in a Powerwave X spectrophotometer using Kineticalc-KC4 software – version 2.5 rev 17.

3.2.5 Cholesterol

Serum cholesterol was measured using a Sigma Cholesterol Kit #402-20. Standards were made using cholesterol Calibrator # C 7921 (Sigma Diagnostics) to produce a linear response curve and the controls were prepared using Accuset Liquid

Calibrator A2539 (Sigma Diagnostics). Once thawed, samples were diluted by mixing 15 μl of sample serum with 45 μl of saline. 10 μl of the diluted samples, standards and controls were placed in duplicate into each well. To each well, 200 μl of reagent was added and the plate was incubated at 37°C for 5 minutes. During this time, cholesterol esters are enzymatically hydrolyzed by cholesterol esterase that is found in the reagent to form cholesterol and free fatty acids. The free cholesterol is then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydrobenzoic acid and 4-aminoantipyrine in the presence of peroxidase to form chromophore, a dye that can be quantified at 500-550 nm. After incubation the absorbance was read at 500 nm by a Powerwave X spectrophotometer using Kineticalc-KC4 software – version 2.5 rev 17 to determine total serum cholesterol levels.

3.3 Lipid Analysis

Lipids were analyzed using a modified Folch extraction method (Folch, 1957). An approximate one gram section of frozen liver and a quarter section of the left kidney were used for fatty acid analysis and exact weighs were measured prior to analysis. The fatty acids that were analyzed were those of carbon length 16 to 24, as these fatty acids are components of the unsaturated fatty acid metabolism pathway.

3.3.1 Extraction

100 μL of C17 (16 mg/ml heptadecosanoic acid (H-3500, Sigma Diagnostics, Oakville, On) in chloroform) was added to each sample to act as an internal standard. To the standard, a 0.5 to 1.0 gram sample and 10 ml of 2:1 chloroform:methanol containing

0.01% of butylated hydroxytoluence (Sigma Diagnostics, B-1378) were added to the tube. The mixture was homogenized using a Brinkman Polytron Homogenizer (Switzerland) until the liquid had a uniform consistency. The rotor was rinsed with 3 ml (2 x 1.5 ml) of methanol (Optima Grade A454-4, Fisher Scientific, Nepean, Ontario) into the homogenization tube; then capped and vortexed (Maxi Mi II –Type 37600 Mixer, Dubuque, Iowa) for 15 seconds.

The sample was placed in a Beckman GP Centrifuge and centrifuged for 10 – 15 seconds at 800 *g* to separate the solvent and tissue. Using a Pasteur pipet, the solvent layer was transferred into a clean 20 ml screw top tube. To the solvent layer, 6 ml of chloroform (Optima Grade C297-4, Fisher Scientific) and 5 ml of 0.73% sodium chloride solution were added. The tubes were capped and vortexed for 30 seconds and then recentrifuged for another 5 – 10 minutes.

The top layer was removed and discarded. Tube sides were carefully rinsed with 1 – 2 ml of theoretical upper phase (chloroform: methanol: water 3:48:47) and then the top layer was removed and discarded. This procedure was repeated a second time. The bottom layer was placed in a 15 ml screw top tub and evaporated to dryness under nitrogen in a Meyer N-Evap Analytical Evaporator (Organomation Associate Inc, South Berlin, Massachusetts) waterbath at a temperature of 30°C. Once the solvent had evaporated, the tubes were washed with 2 x 1 ml of chloroform and transferred to a 4 ml screw top tube with a Teflon lid. The tubes were flushed with nitrogen and stored in a –40°C freezer for later methylation.

3.3.2 Methylation

From the previously extracted lipid, 500 μl of lipid containing solvent was transferred into a 8 ml screw top tube and then evaporated to dryness under nitrogen in a 30°C waterbath. A volume of 1 ml of toluene (HPLC Grade T290-1, Fisher Scientific) and 1.2 ml of methanolic HCL-3N (Supelco, Bellefonte, Pennsylvania) was added to the tubes, capped and then vortexed for 30 seconds. The tubes were placed in a preheated 80 °C Isotemp Oven (Fisher Scientific) for 1 hour to catalyze the methylation process. The tubes were removed and allowed to cool for 10 – 15 minutes. Once cooled, 1 ml of deionized water was added to the tubes, the tubes were capped and vortexed for 15 seconds, then centrifuged for 5 minutes at 800 *g*.

The top layer was transferred to a clean 8 ml tube. To the bottom layer, 1 ml of petroleum ether (Optima Grade E 120-4, Fisher Scientific) was added. The tube was again capped and vortexed for 15 seconds and recentrifuged for 5 minutes. Again the top layer was removed and combined with the previously removed top layer. To the combined top layers 2 ml of deionized water was added and the tubes were vortexed and centrifuged as above.

Half of the top layer was removed and placed in a Targer DG GC vial (National Scientific Company, Jeddah, Saudi Arabia) and dried under nitrogen in a dry bath. Once the solvent had partially evaporated, the rest of the top layer was transferred into the vial to be evaporated. Once the solvent had evaporated, 700 μl of heptane (HPLC Grade H 350-1, Fisher Scientific) was added to the tubes, the tubes were flushed with nitrogen and then capped. Samples were stored in a –40°C freezer for analysis using gas chromatography.

3.3.3 GC Analysis

Measurement of methyl esters of fatty acids ranging from C:16 to C:24 was achieved using a Varion Star 3400 GC instrument, with a J & W DB 225 30 meter column. Injection of 0.5 μl of sample was used with a split ratio of 10. C17 was used as a standard and peaks were identified using commercial standards. Chromatographs were interpreted using Varian GC Star software, version 3.0. The fatty acids that calculated the % of total fatty acid content were: 16:0, 16:1, 18:0, 18:1 *cis 9,* 18:1 *cis 11*, 18:3 (n-6), 18:3 (n-3), 20:0, 20:1, 20:2, 20:3n-6, 20:3n-6, 20:4n-6, 20:5, 21:0, 22:0, 22:1, 22:2, 22:5, 22:6 23:0, 24:0, and 24:0. The total percent of each major fatty acid between C:16 and C:24 was calculated by the ratio of a particular fatty acid to total fatty acids in that range.

3.4 Renal PGE_2 Assay

On the day of necropsy, the lower third of the right kidney was obtained, weighed and placed in 10 mL of 10 fold diluted Hanks Balanced Salt Solution (Sigma H 4385) for ex vivo release of PGE_2. The tissue sample was then homogenized using a Polytron, Brinkmann Homogenizer and incubated at 37°C for one hour in a Fisher Scientific Shaking Waterbath (model 1125). After incubation, 100 μL of indomethocin (10 mmol/ml in ethanol - I-7378, Sigma Diagnostics) a COX inhibitor, was added to the homogenate to prevent further prostanoid release. The sample was vortexed using a Max Mix II to distribute the indomethocin. The sample was then centrifuged in a Beckman Avanti J-25 I Centrifuge for 15 minutes at 4°C at 416 *g*. The supernatant was poured off into a clean glass tube and then frozen at –80°C for later PGE_2 analysis.

PGE_2 was measured using a PGE_2 immunoassay kit (R&D Systems, Minneapolis, Minnesota). PGE_2 standard and samples were prepared using a 10 fold dilution. The

microassay plate was labeled for standards and samples and 100 μl of diluted sample and standard was placed into the appropriate well. 50 μl of each PGE_2 conjugate and antibody solution were added to each well. The microplate was then covered with adhesive strip and incubated for 2 hours at room temperature. During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. The samples PGE_2 and the fixed amount of conjugate PGE_2 compete for the sites on the mouse monoclonal antibody.

After 2 hours, the wells were aspirated and washed with 200 μL of wash buffer three times to remove excess conjugate and unbound sample. After removal of all wash buffer, 200 μL of pNPP substrate was added to all wells to determine the bound enzymatic activity and the plate was incubated for 1 hour at room temperature while the colour developed. After 1 hour, 50 μL of stop solution was added to each well to prevent further enzymatic activity. The microplate was then immediately placed into a Sprectra MAX 340 (Molecular Devices, Sunnyvale, CA) spectrophotometer to measure optical density at a wavelength of 405 nm and a correction set at 580 nm. The software program used was SOFTmax © Pro, Version P1.12, (Molecular Devices Corp., Sunnyval, California).

This particular assay demonstrates a sensitivity of less than 36.2 pg/ml. This kit does not have specificity for PGE_2 and demonstrates cross reactivity to a number of compounds. In particular, it shows cross reactivity towards PGE_1 (70%) and PGE_3 (16.3%). The cross reactivity with PGE_1 is relatively high. This is of little concern as there is little to no PGE_1 in normal functioning renal tissues. Although no research has proven or disproved it, the possibility of a shift in PG production in renal disease may

result in a higher PGE_1 level resulting in false alteration in PGE_2 levels. Further studies are required in this area.

3.5 Statistical Analysis

Statistical analysis was conducted using the SPSS software program (SPSS Inc, Version 10.0.1, Chicago, Illinois). All variables were subjected to a 2x2x2 Univariate Analysis of Variance. The three independent variables were: 1) diet (soy protein vs casein protein); 2) disease (normal animals vs polycystic animals); 3) feeding time (1 week of feeding versus 3 weeks of feeding). Tests were done for homogeneity of variance using the Levene Test of Equality of Error Variances. For those variables not demonstrating homogeneity, logarithms were taken to produce a normal distribution of variances. In the case of two or three way interactions, Tukey's b post hoc test was used to determine where differences between groups were. Differences were deemed significant at a $P<0.05$.

An outlier's tests was conducted on each parameter using this software program to determine any points that were outside the three standard deviation mark. These values were omitted from the respective group and were not part of the statistical analysis.

4 Results

Of the 87 Han:SPRD-*cy* male rats obtained for this study, 45 animals were placed on the 1-week feeding trial and 42 animals were subjected to the 3 weeks of feeding. Of the animals fed for 1 week, 6 normal and 16 diseased animals were placed on the casein diet, while 5 normal and 18 diseased animals were placed on the soy diet. Of the animals on the 3 week feeding trial, 8 normal and 14 affected animals were placed on the casein diet and 9 normal and 11 affected animals were placed on the soy diet.

The unequal distribution of normal to diseased animals is due to the genetic inheritance pattern of this disease. The disease is not detectable at weanling and diagnosis was made upon termination. For completeness, complete raw data presented in this section as a figure can be found in the appendix section.

4.1 Assessment of Growth

Daily food intake of animals of both diets can be found in Figure 3. Soy fed animals had a slightly higher daily intake then casein fed animals despite the fact that casein fed animals were pair fed against soy fed animals. Body weight, femur length and femur weights were all used as indicators of animal growth. As demonstrated in Table 3, all three parameters showed no effect of diet or disease status. However, a significant difference was detected between the two feeding times in all three indices. The animals experienced a 97% increase in body weight from 1 week of feeding to 3 weeks of feeding (99.0 ± 4.4 to 194.8 ± 4.6g, $P<0.001$). After 3 weeks of feeding, femur length (2.86 ± 0.03 vs 2.27 ± 0.03cm, $P<0.001$) and femur weight (0.56 ± 0.01 vs 0.31 ± 0.01 g, $P<0.001$) were 26% and 80% higher than those values seen after 1 week of feeding.

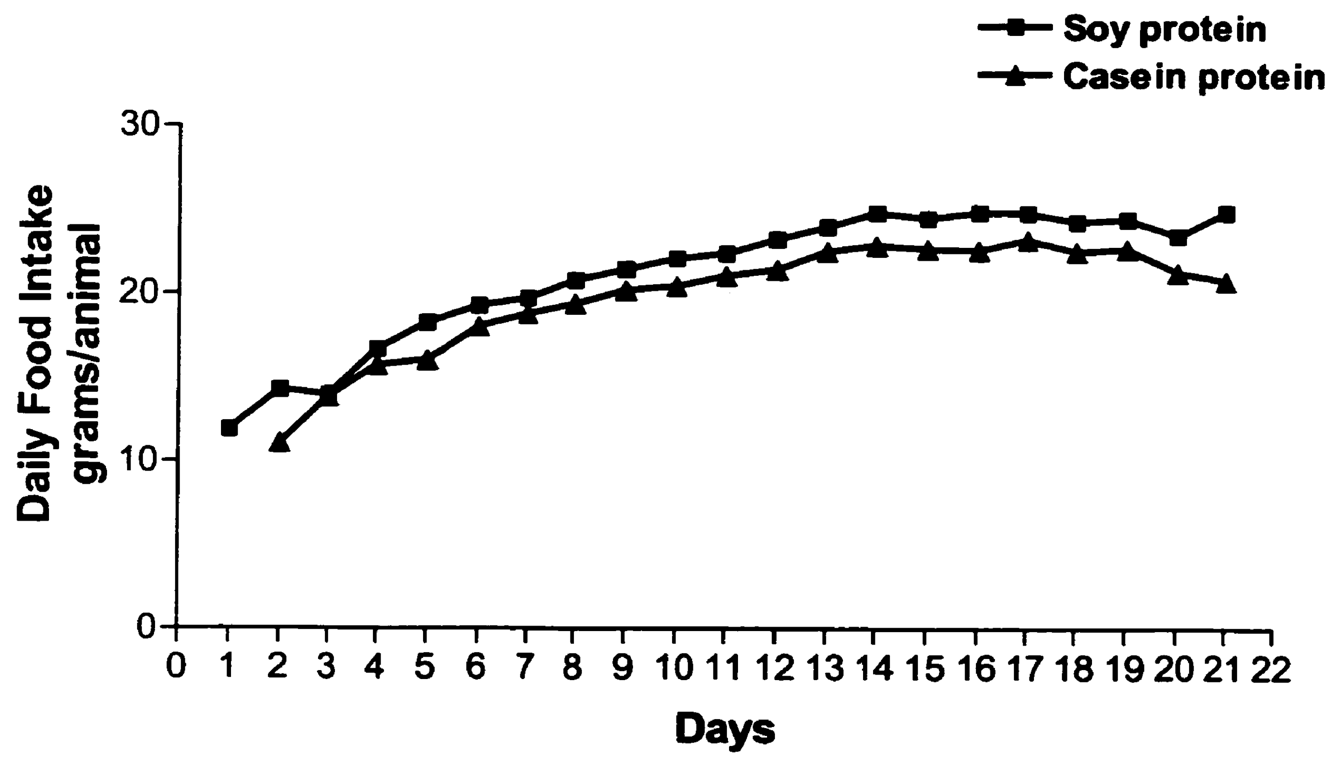

Figure 3 Average daily food intake of animals fed soy and casein for 3 weeks. Data is expressed as mean ± SEM (N=41).

Table 3 Indicators of growth from normal or affected Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
n Value (N=87)	6	5	16	18	8	9	14	11	NA	NA	NA	NA	NA	NA	NA
Weight (g)	96.6 (5.7)	99.3 (5.7)	101.9 (3.5)	98.0 (3.4)	192.7 (5.0)	196.2 (4.7)	192.6 (3.7)	197.8 (4.2)	NS	0.000	NS	NS	NS	NS	NS
Femur Length (cm)	5.84 (0.08)	5.72 (0.10)	5.79 (0.08)	5.66 (0.05)	7.31 (0.08)	7.26 (0.08)	7.21 (0.08)	7.24 (0.08)	NS	0.000	NS	NS	NS	NS	NS
Femur Weight (g)	0.31 (0.02)	0.32 (0.02)	0.32 (0.01)	0.30 (0.01)	0.57 (0.01)	0.56 (0.01)	0.55 (0.01)	0.54 (0.01)	NS	0.000	NS	NS	NS	NS	NS

Data presented as mean ± SEM
NS, data not significantly different. NA, not applicable

4.2 Disease Progression

Figure 4 illustrates the difference detected in the average kidney weight (per 100 g body weight) which demonstrated a diet by time by disease interaction (P=0.003). After 1 week of feeding, the diseased animals had a 27 % higher kidney weight than the normal animals, with no effect of diet between each group. Similar results were detected after 3 weeks of feeding with the casein fed diseased animals having an 87 % higher kidney weight than the normal animals, with no diet effect seen between the normal animals. However, the diseased soy fed animals had a 37% higher average kidney weight over the normal animals and a 27 % less total kidney weight when compared to the casein fed diseased animals. With respect to time, normal animals at 1 week had a higher average kidney weight per body weight than those seen at 3 weeks. The diseased animal kidney weights remained constant over time with the exception of the soy fed animals after 3 weeks of feeding having a lower kidney weight than its counterparts.

In cyst volume and renal fibrous volume, only the diseased animals were measured, as normal animals do not have cystic kidneys. Cyst volume demonstrated a diet by time interaction (Figure 5). After 1 week of feeding, there was no effect of diet. There was however, a diet effect detected between casein and soy fed animals after 3 weeks of feeding. Soy fed animals had a 38% lower renal cyst volume than the casein fed animals (0.13 ±0.01 ml/kg vs 0.21 ±0.02 ml/kg body weight, P<0.001). Cyst volume relative to body weight was 50 % lower in the soy fed animals after 3 weeks than after 1 week of feeding. There was no significant change in cyst volume in casein fed animals over time. Figures 6 and 7 are representative sections of renal tissue from casein and soy fed diseased animals, respectively, after 3 weeks of dietary intervention.

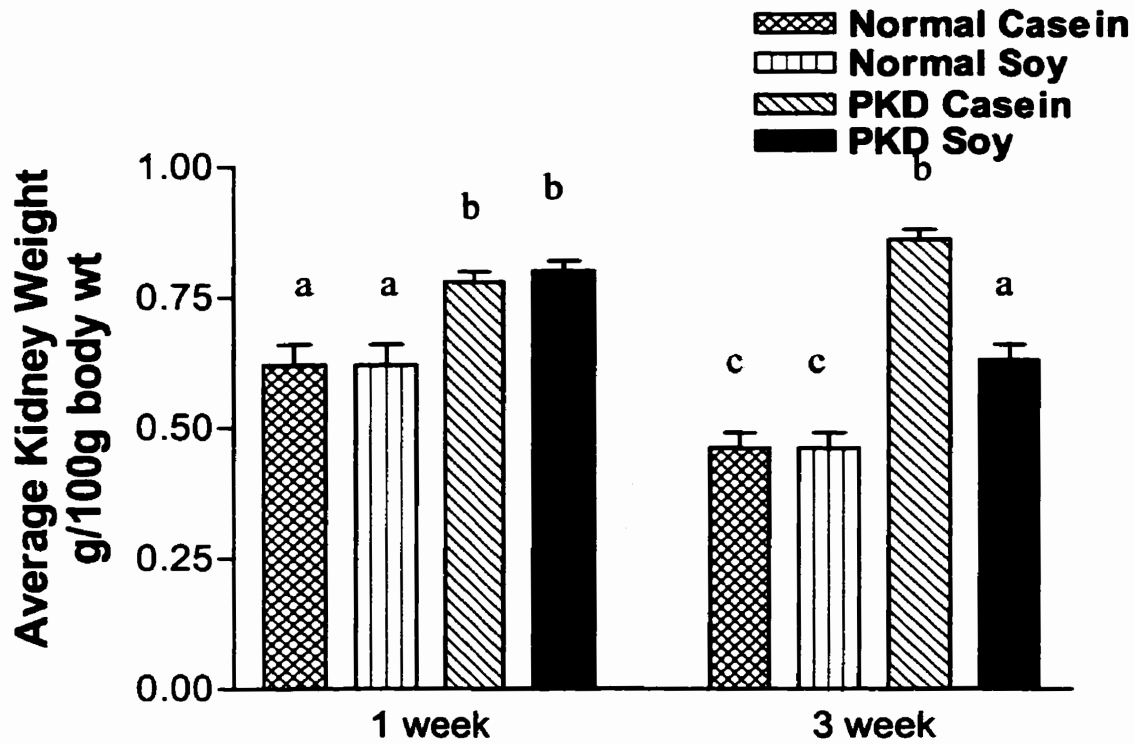

Figure 4 Average kidney weight for diseased and normal Han:SPRD-*cy* rats fed soy or casein protein for 1 or 3 weeks. Data is expressed as mean ± SEM (N=87). Columns with different letters are significantly different at $P<0.05$ using Tukey's b post hoc test. Time by diet by disease interaction, P=0.003.

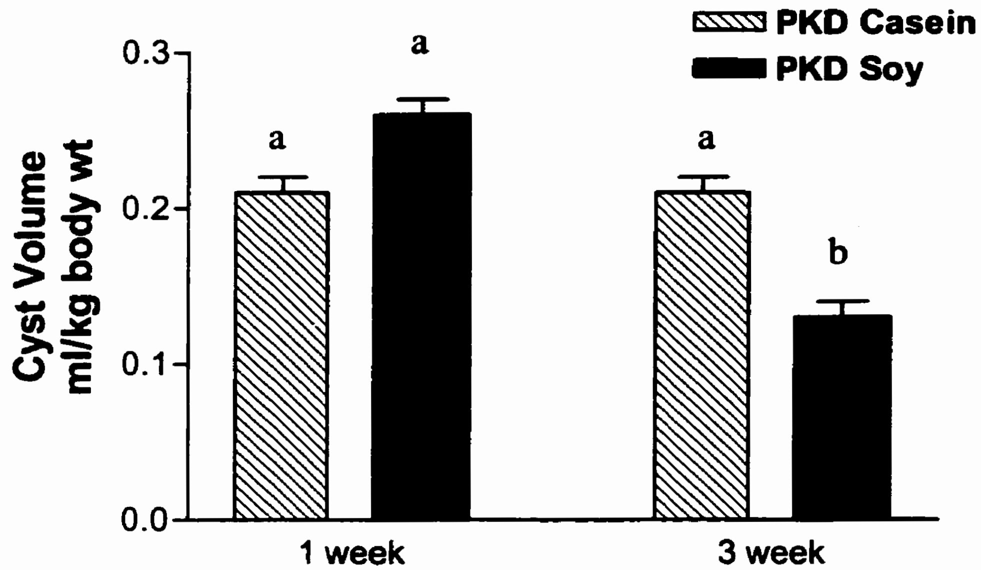

Figure 5 Renal cyst volume in affected Han:SPRD-*cy* rats fed soy or casein protein for 1 or 3 weeks. Data are expressed as mean ± SEM (N=59). Columns with different letters are significantly different at $P<0.05$ using Tukey's b post hoc test. Diet by disease interaction, $P=0.001$.

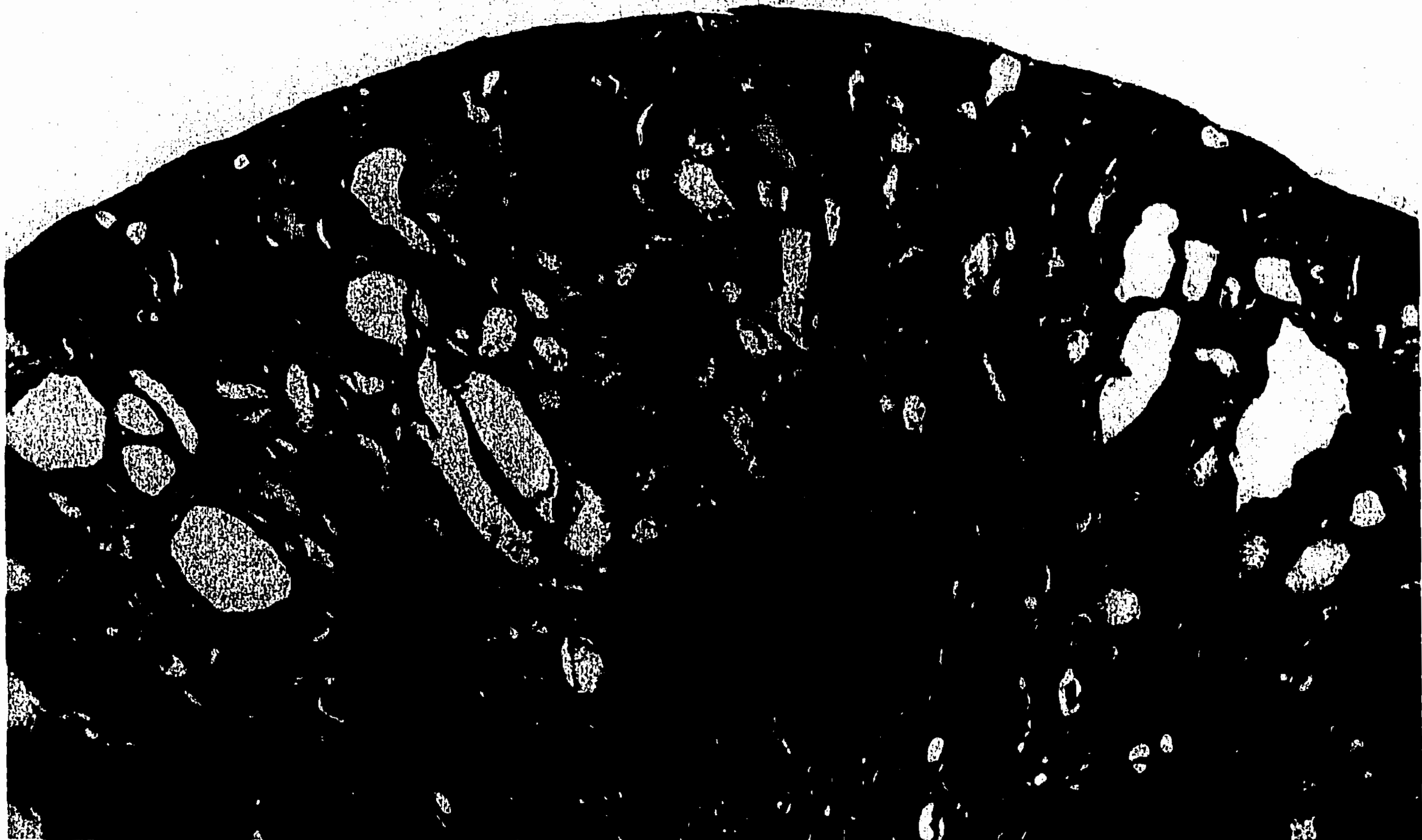

Figure 6 Kidney section from affected Han:SPRD-*cy* rat fed 20% casein for 3 weeks post weaning (hematoxylin and eosin, 2X objective, 30 fold magnification).

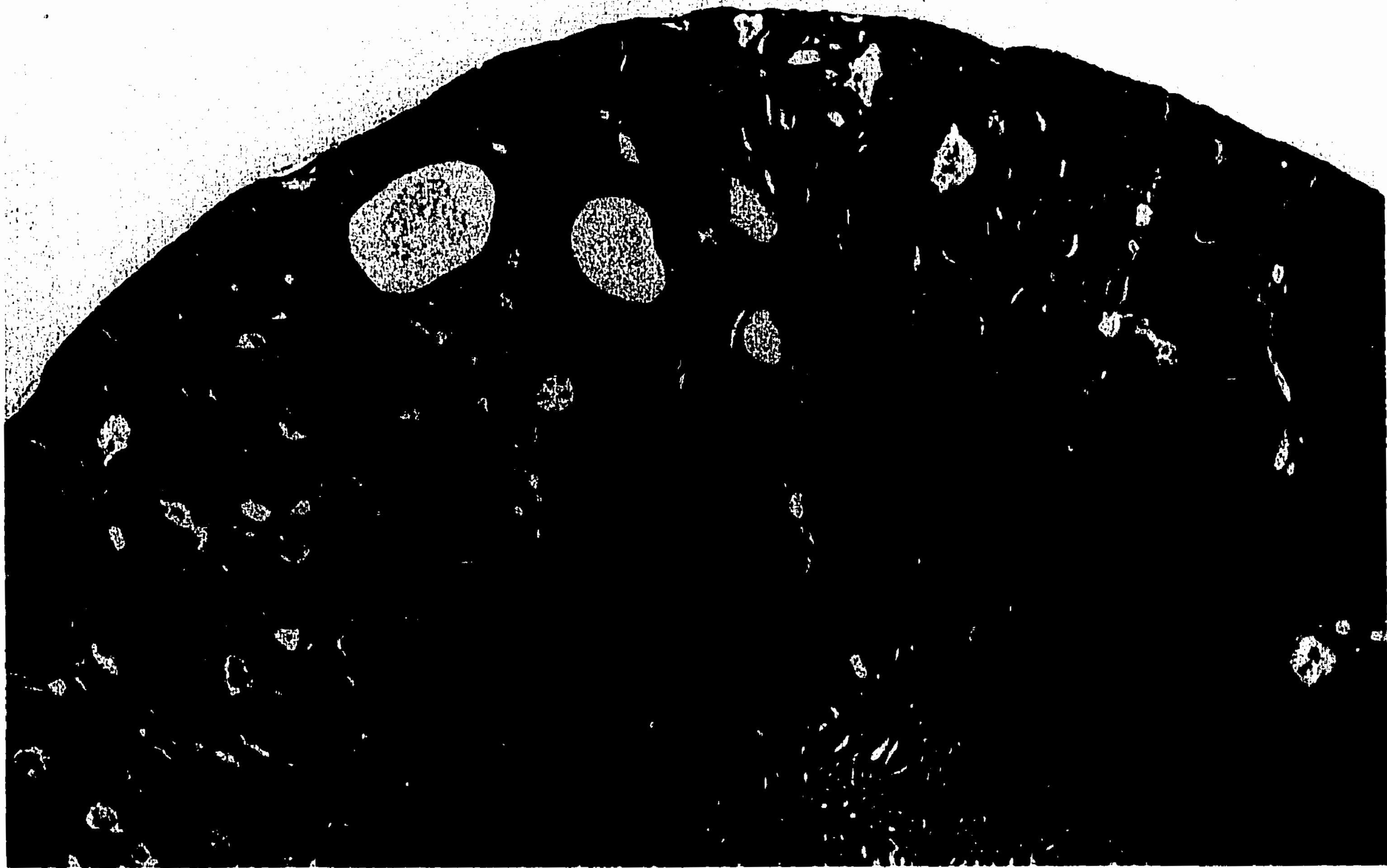

Figure 7 Kidney section from affected Han:SPRD-*cy* rat fed 20% soy protein for 3 weeks post weaning (hematoxylin and eosin, 2X objective, 30 fold magnification)

In renal fibrosis, main effects of both time and diet were observed (Figure 8). Over time, the animals fed for 3 weeks had just over one third (36%) of the fibrous volume seen after 1 week of feeding (0.05 ● 0.01 vs 0.14 ● 0.01 ml/kg, $P<0.001$). Diet played a role in renal fibrous volume with soy protein lowering fibrous volume by 20% when compared to the casein fed animals. Figures 9 and 10 are representative sections of renal tissues stained with aniline blue from casein and soy fed animals, respectively, after 3 weeks of dietary intervention.

4.3 Biochemistry

Creatinine clearance is often used as a biological marker for kidney function (Gabow, 1990, Ogborn et al, 1995) and is an estimate of GFR. A time by diet interaction was detected in creatinine clearance (Table 4). Over time, both normal and diseased animals fed casein remained constant but soy fed animals demonstrated a 2.03 and 2.10 times higher creatinine clearance after 3 weeks when compared to 1 week of feeding. The only diet effect with respect to creatinine clearance was detected between the diseased animals after 3 weeks of feeding. The diseased animals fed casein protein for 3 weeks had a 37 % lower creatinine clearance than the diseased animals fed soy protein (3.48 ± 0.41 vs 5.39 ± 0.49 mL/min/kg, $P<0.05$). There was no effect of disease in creatinine clearance levels.

A diet by time by disease interaction occurred in serum triglyceride analysis (Table 4). In regard to diet, serum triglycerides only demonstrated a difference between the casein and soy fed animals in the diseased animals after 1 week of dietary

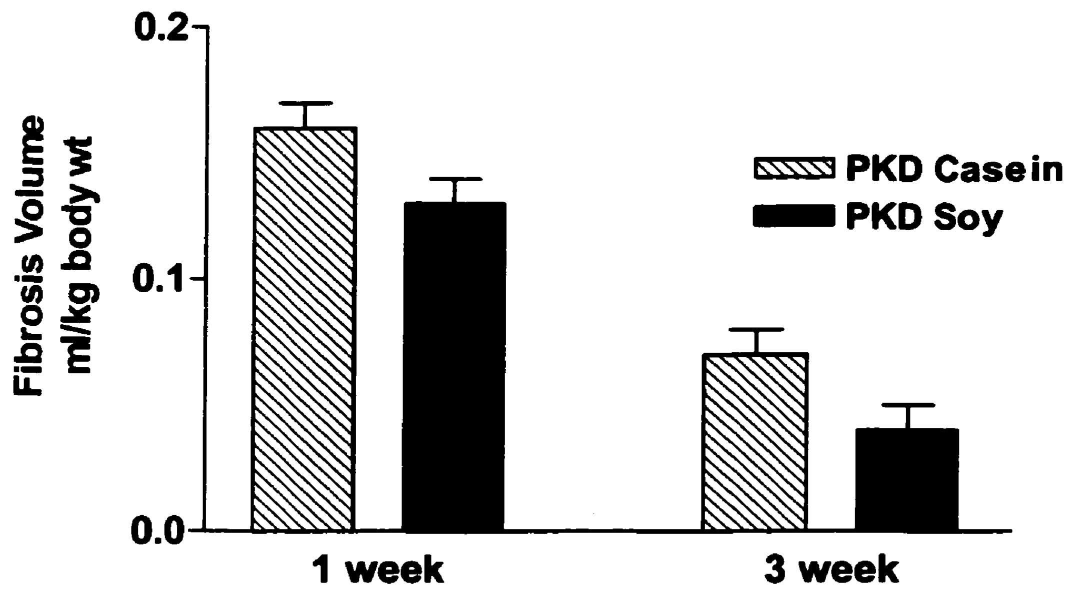

Figure 8 Renal fibrous volume in affected Han:SPRD-*cy* rats fed soy or casein protein for 1 or 3 weeks. Data are expressed as mean ± SEM (N=59).

<u>Main Effects</u>: diet, P=0.025; feeding time, P=0.000

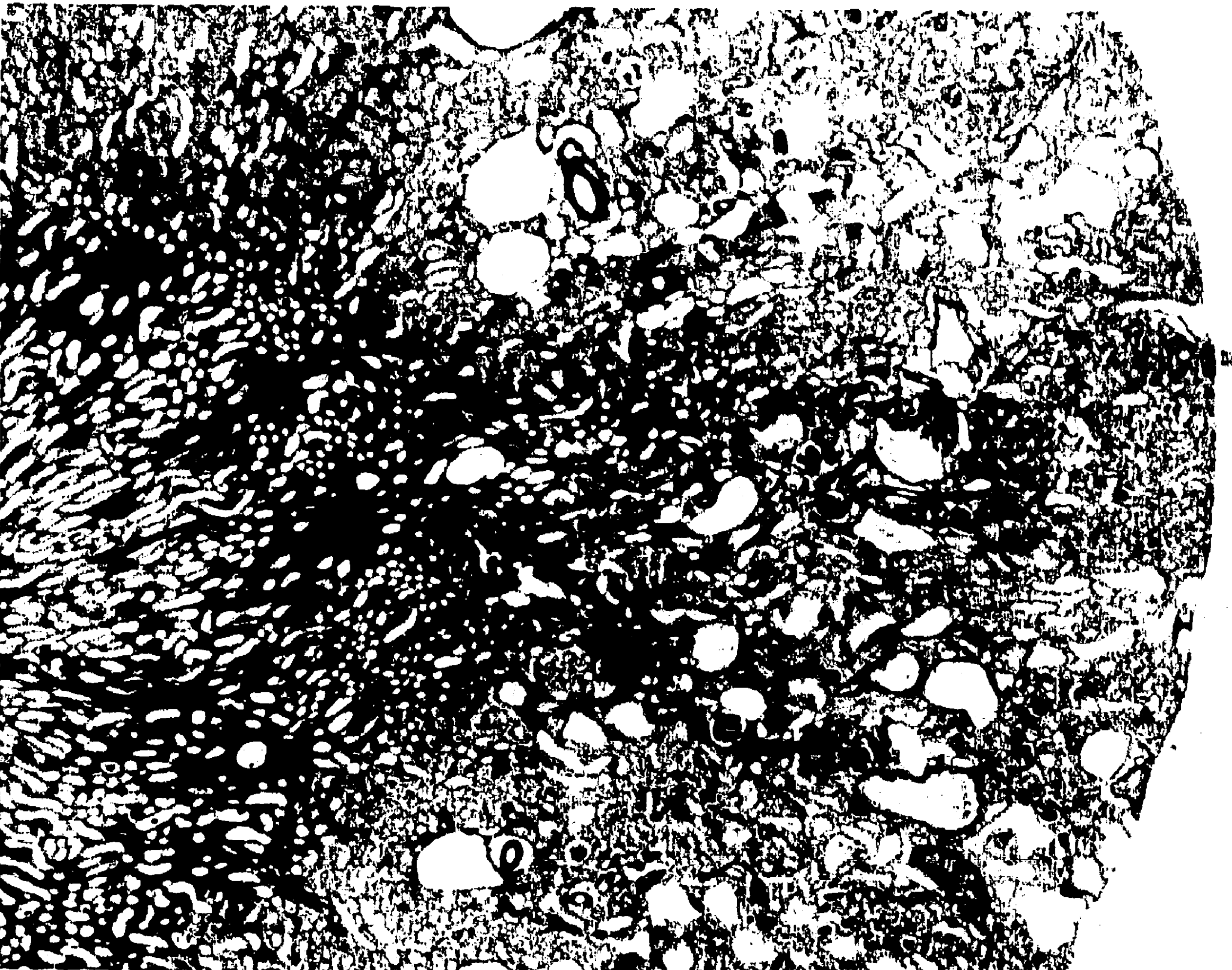

Figure 9 Kidney section from affected Han:SPRD-*cy* rat fed 20% casein for 3 weeks post weaning (aniline blue, 2X objective, 30 fold magnification). Darker areas represent collagen III, a component of fibrotic tissue.

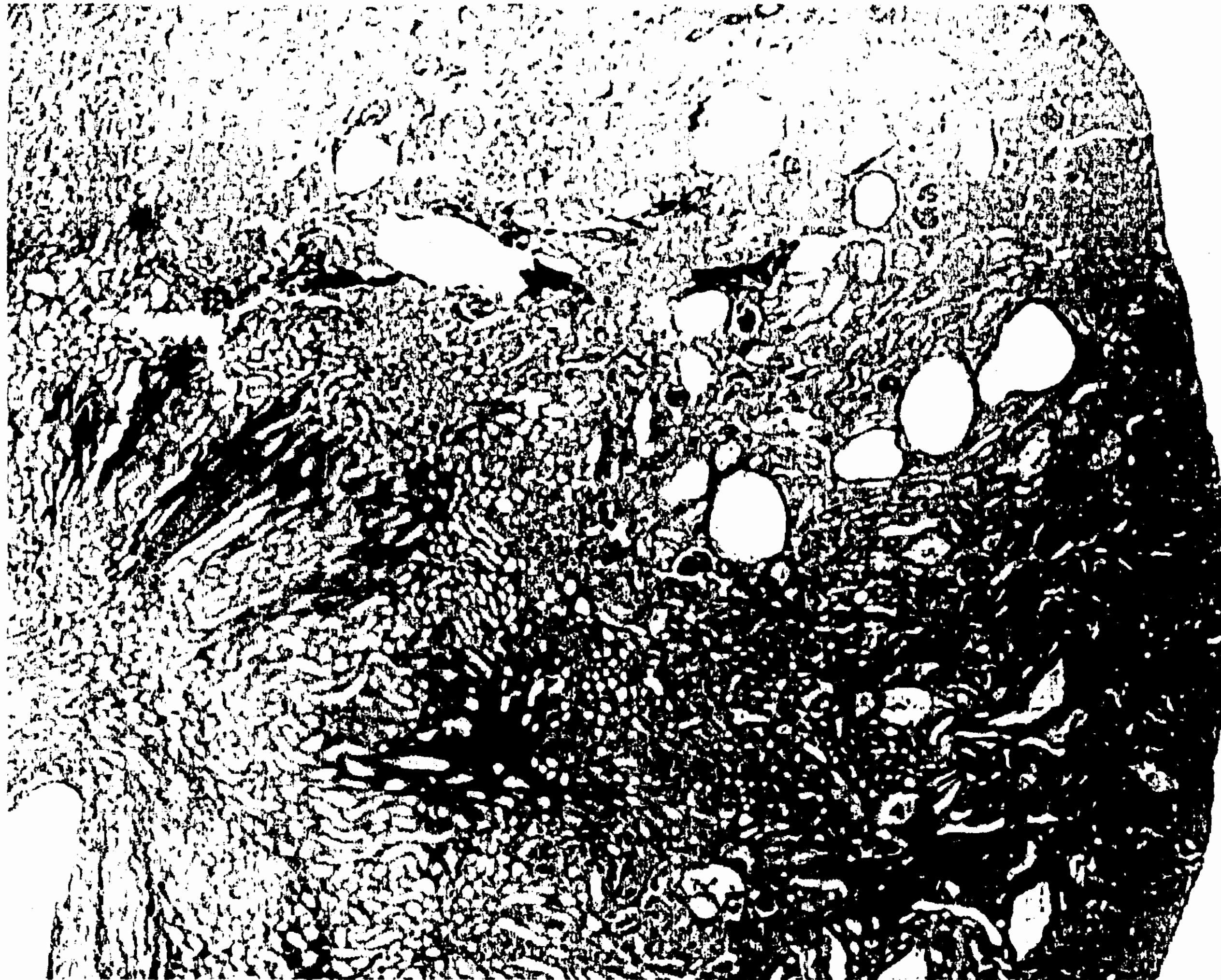

Figure 10 Kidney section from affected Han:SPRD-*cy* rat fed 20% soy protein for 3 weeks post weaning (aniline blue, 2X objective, 30 fold magnification). Darker areas represent collagen III, a component of fibrotic tissue.

Table 4 Serum biochemistry from normal or affected Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Serum Creatinine (μmol/L)	47.65 [ab] (4.30)	45.40 [a] (4.30)	48.66 [a] (2.63)	54.54 [ab] (2.55)	48.69 [ab] (3.72)	62.21 [b] (3.72)	55.89 [ab] (2.81)	57.09 [ab] (3.18)	NS	0.004	NS	NS	NS	NS	0.033
Creatinine Clearance (mL/min/kg)	2.97 [a] (0.69)	2.62 [a] (0.63)	3.39 [a] (0.40)	2.81 [a] (0.38)	4.61 [ab] (0.59)	5.34 [b] (0.55)	3.42 [a] (0.41)	5.39 [b] (0.47)	NS	0.000	NS	NS	NS	0.017	NS
Cholesterol (mol/L)	4.12 (0.54)	3.59 (0.54)	4.28 (0.33)	4.16 (0.32)	5.35 (0.47)	5.12 (0.47)	4.21 (0.35)	4.69 (0.40)	NS	0.011	NS	NS	NS	NS	NS
Triglyceride (mol/L)	0.19 [ab] (0.05)	0.15 [a] (0.05)	0.12 [a] (0.03)	0.30 [b] (0.03)	0.23 [ab] (0.04)	0.28 [b] (0.04)	0.28 [b] (0.03)	0.29 [b] (0.03)	NS	0.002	NS	NS	NS	NS	0.014

Data presented as mean ± SEM (N=87).
Groups with different letters in rows are significantly different at $P<0.05$ by Tukey's b post hoc test.
NS, not significantly different.

intervention. The diseased animals fed soy protein had a 2.5 times higher serum triglyceride over the diseased animals fed casein. The normal animals fed soy protein demonstrated a time effect with the animals fed for 3 weeks having a 87 % higher triglyceride level than the 1 week fed animals. The diseased animals fed casein also demonstrated a time effect, having a 130 % higher serum triglyceride level after 3 weeks when compared to after the 1 week fed animals. Disease played a role in the soy fed animals after 1 week of feeding only. The normal animals fed soy protein had half of the serum triglyceride level of the affected animals.

An overall time effect was detected in serum cholesterol levels with the animals fed for 3 weeks having a 20% higher cholesterol level (4.84 ± 0.42 vs 4.02 ± 0.43 mmol/L, $P<0.011$) than animals fed for 1 week. There was no effect of disease or diet in serum cholesterol levels.

4.4 Fatty Acids

Fatty acid analysis was conducted to examine mainly long chain fatty acids and only those fatty acids of carbon length 16 to 24 were measured. A cut off of at least 1 % of total fatty acid composition was used.

A representative chromatograph for kidney and liver tissues can be found in Figure 11 and 12 respectively. The chromatograph identifies the peaks of interest, indicates when each fatty acid went through the column and illustrates the amount of each peak. For simplicity, fatty acids were broken down into the following categories: saturated, monounsaturated and polyunsaturated fatty acids.

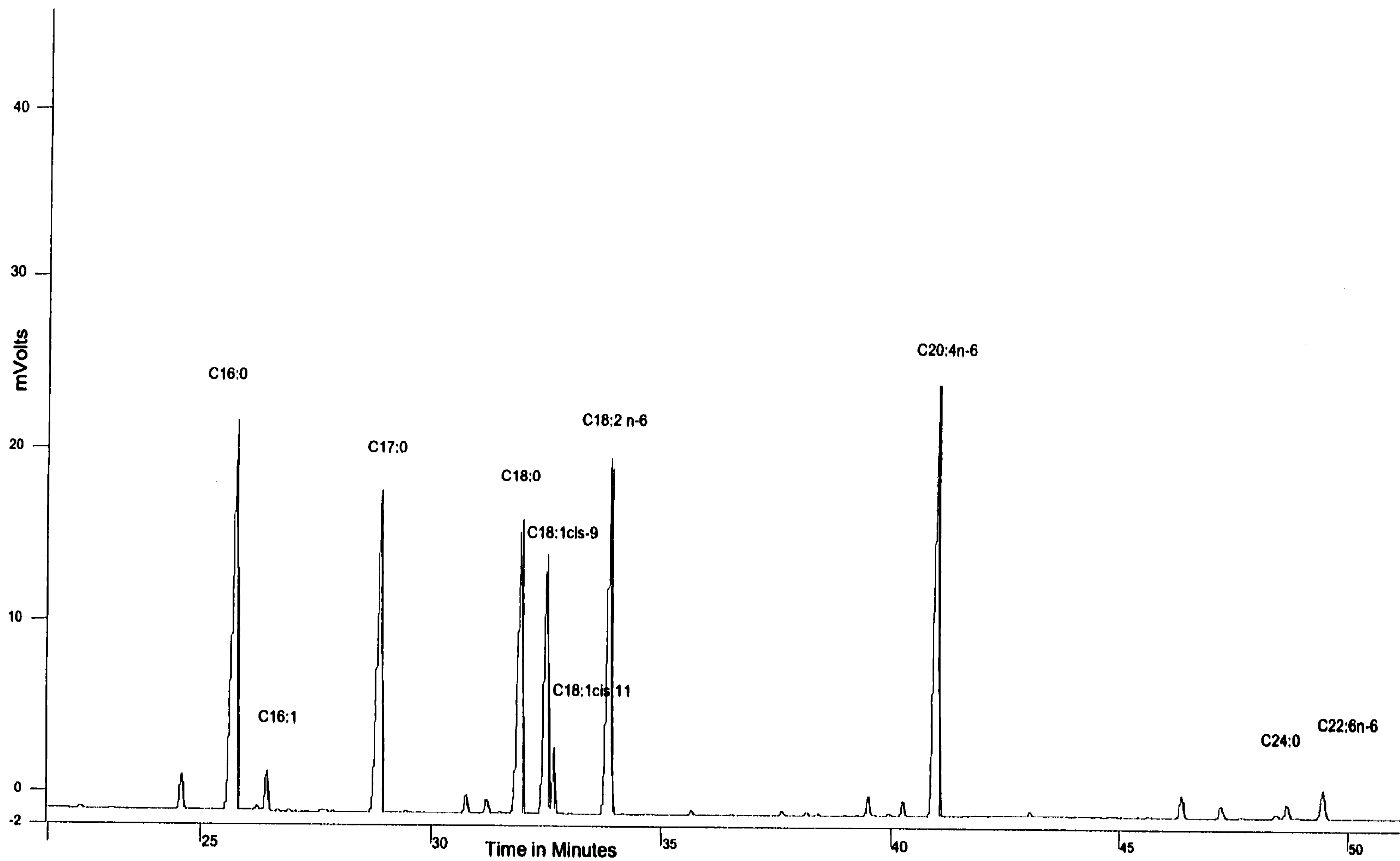

Figure 11 Representative chromatograph of kidney tissue after 3 weeks of feeding.

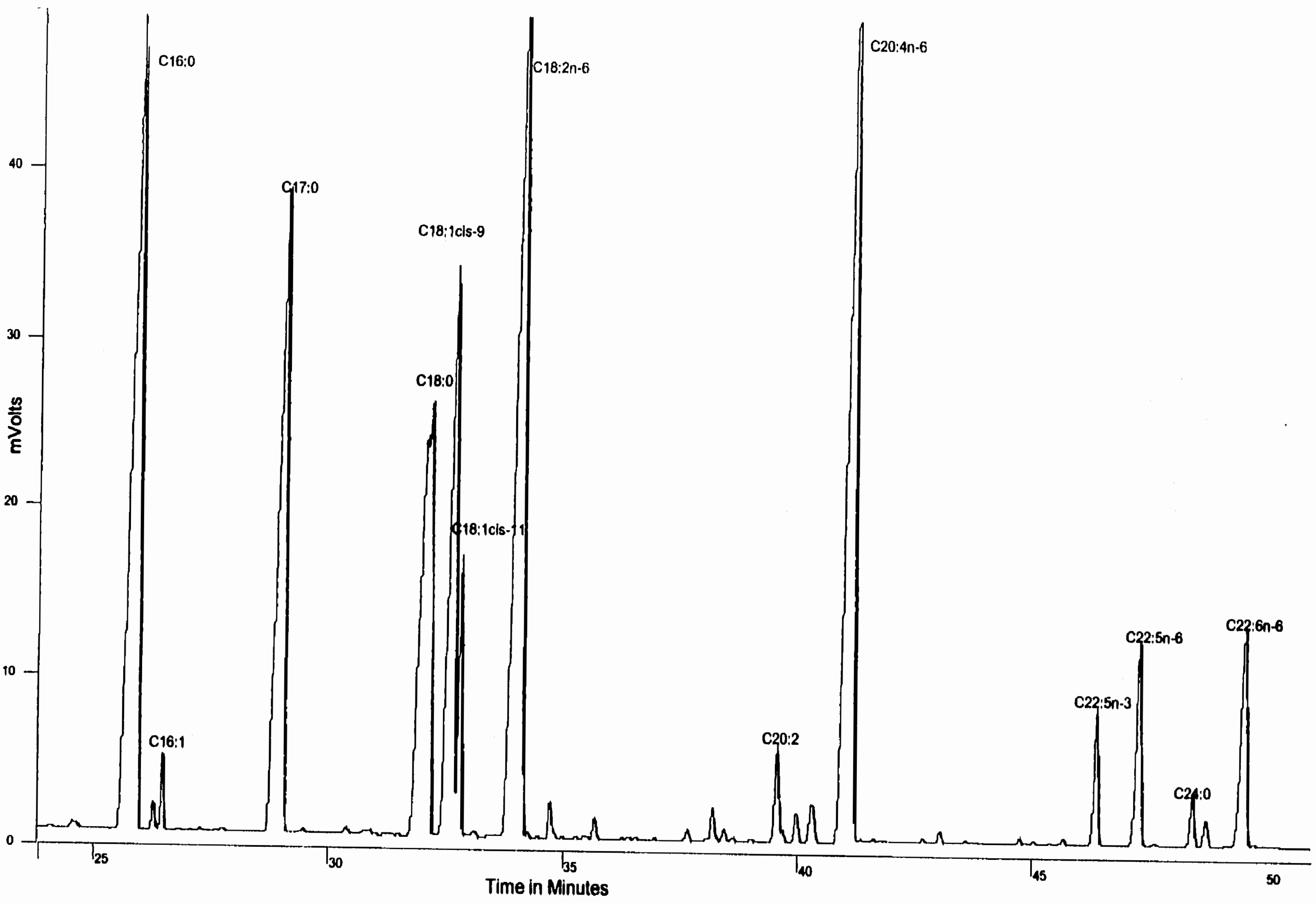

Figure 12 Representative chromatograph of liver tissue after 3 weeks of feeding.

4.4.1 Saturated Fatty Acids

The saturated and monounsaturated fatty acids in the kidney can be found in Table 5 and those in the liver can be found in Table 6.

4.4.1.1 Palmitic Acid 16:0

Although there was a time by disease interaction detected in renal tissues, there was not a significant difference between normal and diseased animals or after 1 and 3 weeks of dietary intervention. A main effect of diet on palmitic acid was detected with the animals fed soy protein having a lower (22.50 ± 0.55 % by weight) palmitic acid composition than the casein fed animals (23.85 ± 0.54 % by weight) (P=0.001).

An overall diet effect was detected in hepatic tissue, with soy protein feeding having a 5% lower palmitic acid content than casein fed animals (19.56 ± 0.26 % vs 20.6 ± 0.26 % by weight, P=0.012).

4.4.1.2 Stearic Acid 18:0

Renal stearic acid content demonstrated a time by disease interaction (P=0.045). Normal animals fed soy protein were the only group of animals to demonstrate a time effect. The normal soy fed animals after 3 weeks of feeding had a 15 % higher stearic acid composition (18.21 ± 0.54 %) than the 1 week feeding group (15.77 ± 0.73 %) (P<0.05).

An overall time effect was shown in hepatic tissues. Stearic acid content decreased from 1 to 3 weeks of feeding with the animals on the 1 week feeding trial having a 26 % higher stearic content (16.26 ± 0.37 %) than the animals on the 3 week feeding trial (12.87 ± 0.35 %) (P=0.000). A main effect of diet was also discovered in

Table 5 Kidney saturated and monounsaturated fatty acids from normal or affected Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
Fatty Acid (% w/w)	Normal		Affected		Normal		Affected								
	Casein	Soy	Casein	Soy	Casein	Soy	Casein	Soy	Diet	Time	PKD	Time x PKD	Diet x PKD	Time x Diet	Time x Diet x PKD
Palmitic Acid 16:0	24.04 [ab] (0.68)	23.05 [ab] (0.75)	23.66 [ab] (0.43)	22.19 [a] (0.40)	23.02 [ab] (0.59)	22.15 [a] (0.56)	24.66 [b] (0.45)	22.61 [ab] (0.50)	0.001	NS	NS	0.036	NS	NS	NS
Palmitoleic Acid 16:1	1.14 [ab] (0.33)	1.21 [ab] (0.36)	1.27 [ab] (0.21)	0.79 [a] (0.19)	1.12 [ab] (0.28)	0.80 [a] (0.28)	1.76 [b] (0.21)	1.15 [ab] (0.24)	NS	NS	NS	0.033	NS	NS	NS
Stearic Acid 18:0	16.57 [ab] (0.66)	15.77 [b] (0.73)	16.21 [ab] (0.42)	17.18 [ab] (0.38)	17.23 [ab] (0.57)	18.21 [a] (0.54)	15.96 [b] (0.43)	17.51 [ab] (0.49)	NS	0.035	NS	0.045	NS	NS	NS
Oleic Acid 18:1 *cis 9*	9.36 (1.35)	12.03 (1.48)	11.04 (0.85)	9.59 (0.78)	9.41 (1.17)	8.76 (1.10)	12.2 (0.88)	9.49 (1.00)	NS	NS	NS	NS	NS	NS	NS
Vaccenic Acid 18:1 *cis 11*	4.04 (0.52)	2.41 (0.57)	2.96 (0.33)	2.62 (0.30)	2.66 (0.45)	2.32 (0.43)	2.53 (0.34)	3.14 (0.38)	NS	NS	NS	NS	NS	NS	NS
Lignoceric Acid 24:0	1.10 (0.09)	1.24 (0.10)	1.17 (0.06)	1.17 (0.06)	1.22 (0.08)	1.09 (0.08)	1.08 (0.06)	1.04 (0.07)	NS	NS	NS	NS	NS	NS	NS

Data presented as mean ± SEM (N=87), NS data not significantly different.
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test.

Table 6 Liver saturated and monounsaturated fatty acids from normal or affected Han:SPRD-*cy* rats fed casein or soy protein diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
	Normal		Affected		Normal		Affected								
Fatty Acid (% w/w)	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Palmitic Acid 16:0	20.60 (.44)	19.91 (0.55)	20.44 (0.31)	20.23 (0.29)	20.80 (0.44)	19.48 (0.41)	20.40 (0.33)	19.26 (0.37)	0.012	NS	NS	NS	NS	NS	NS
Palmitoleic Acid 16:1	1.49 (0.17)	0.58 (0.18)	1.49 (0.10)	0.70 (0.10)	1.66 (0.15)	0.82 (0.14)	1.34 (0.11)	0.70 (0.12)	0.000	NS	NS	NS	NS	NS	NS
Stearic Acid 18:0	16.20 (0.45)	16.48 (0.50)	15.86 (0.28)	16.49 (0.26)	11.82 (0.39)	13.35 (0.37)	12.63 (0.30)	13.69 (0.34)	0.001	0.000	NS	NS	NS	NS	NS
Oleic Acid 18:1 *cis 9*	11.18 (0.79)	8.69 (0.86)	11.82 (0.48)	8.78 (0.45)	14.41 (0.68)	11.63 (0.64)	13.68 (0.51)	10.61 (0.58)	0.000	0.000	NS	NS	NS	NS	NS
Vaccenic Acid 18:1 *cis 11*	2.38 (0.20)	1.63 (0.21)	2.44 (0.12)	1.65 (0.11)	2.91 (0.17)	2.05 (0.16)	2.32 (0.13)	2.20 (0.14)	0.000	0.003	NS	NS	NS	NS	NS

Data presented as mean ± SEM (N=87)
NS data not significantly different.

hepatic tissues, with the soy fed animals having a 6 % higher amount of stearic acid (15.0 ±0.37%) than the casein fed group (14.12 ● 0.36 %) (P=0.001).

4.4.1.3 Lignoceric Acid 24:0

There were no significant differences found between any of the groups with respect to 24:0 renal content. This fatty acid was below the 1 percent cut off in hepatic tissues and thus not included in the analysis.

4.4.2 Monounsaturated Fatty Acids

The monounsaturated fatty acids that were found in significant amounts were palmitoleic acid (16:1), oleic acid (18:1 *cis 9*) and vaccenic acid 18:1 *cis 11*. There were no overall significant differences found in renal oleic and vaccenic acid and no simple differences found in palmitoleic acid composition that demonstrated a time by disease effect (Table 5).

In hepatic tissue, all three monounsaturated fatty acids demonstrated an overall diet effect. The animals fed soy protein had a 53 %, 22 %, and 26 % lower hepatic composition of palmitoleic acid (0.70 ± 0.14 vs 1.50 ● 0.13 %, P=0.000), oleic acid (9.93 ● 0.63 vs 12.77 ± 0.62 %, P=0.000) and vaccenic acid (1.89 ±0.15 vs 2.57 ● 0.18 %, P=0.000) respectively, when compared to animals fed casein (Table 6). Over time overall oleic composition was higher by 24 %, after 1 week of feeding than (10.12 ± 0.65 % vs 12.53 ± 0.60 %, P=0.000) after 3 weeks of feeding. Vaccenic acid was higher, by 17 %, with the 1 week group having a vaccenic acid content of 2.02 ● 0.16 % and the 3 week fed group having 2.37 ± 0.10 % vaccenic acid content (P=0.003).

4.4.3 Polyunsaturated Fatty Acids

4.4.3.1 Linoleic Acid (LA) 18:2n-6

A diet by time interaction was found in renal tissues (P= 0.035) (Table 7). A diet effect was detected after 1 week of feeding only with normal soy fed animals having a 21 % higher LA levels and diseased animals fed soy protein having a 19 % higher LA composition than their casein fed counterparts after 1 week of feeding (P<0.05). However, the differences were no longer significant after 3 weeks of feeding.

A similar pattern of diet by time interaction was found in the liver (Table 8) (P=0.000). In this tissue, normal animals and diseased animals fed soy protein had a 27 % and 24 % higher LA content than their casein fed counterparts after 1 week of feeding. Again the differences were no longer significant after 3 weeks of feeding. A time effect was detected in casein fed animals only, in both normal and diseased animals. The normal and diseased animals fed casein had a 47 % and 50 % respectively, higher LA content after 3 weeks of feeding when compared to the 1 week feeding trial (P<0.05).

4.4.3.2 Arachidonic Acid (AA) 20:4n-6

As illustrated in Table 7, a time by disease effect occurred in kidney AA (P=0.008). There was no effect of diet in renal AA. Both normal soy and casein fed animals at 1 week were different than their counterparts after 3 weeks of feeding with the 3 week fed animals having a higher AA than the animals fed for 1 week (P<0.05). The only other effect was a disease effect detected between the normal 3 week animals and

Table 7 Kidney polyunsaturated fatty acids from normal or affected Han SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
Fatty Acid (% w/w)	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Linoleic Acid 18:2n-6	13.36 [a] (0.54)	16.17 [b] (0.59)	12.95 [a] (0.34)	15.46 [b] (0.31)	12.85 [a] (0.46)	14.61 [ab] (0.44)	13.71 [a] (0.35)	14.64 [ab] (0.40)	0.000	NS	NS	NS	NS	0.035	NS
Arachidonic Acid 20:4n-6	25.08 [a] (1.52)	21.73 [a] (1.67)	24.86 [a] (1.0)	24.57 [a] (0.88)	28.12 [b] (1.32)	27.41 [b] (1.24)	23.54 [a] (1.0)	25.10 [a] (1.12)	NS	0.027	NS	0.008	NS	NS	NS
Docosahexa-enoic Acid 22:6n-3	2.86 (0.13)	2.69 (0.15)	2.67 (0.09)	2.79 (0.08)	1.74 (0.12)	1.81 (0.11)	1.52 (0.09)	1.71 (0.10)	NS	0.000	NS	NS	NS	NS	NS

Data presented as mean ± SEM (N=87)
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test.
NS data not significantly different.

Table 8 Liver polyunsaturated fatty acids from normal or affected Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
Fatty Acid (% w/w)	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Linoleic Acid 18:2n-6	13.81 [a] (0.65)	17.49 [b] (0.71)	13.83 [a] (0.40)	17.19 [b] (0.37)	20.32 [b] (0.56)	21.48 [b] (0.53)	20.79 [b] (0.42)	20.92 [b] (0.48)	0.000	0.000	NS	NS	NS	0.000	NS
Arachidonic Acid 20:4n-6	19.65 (0.65)	20.94 (0.71)	19.89 (0.40)	20.42 (0.34)	17.48 (0.56)	19.28 (0.53)	17.94 (0.42)	19.70 (0.48)	0.000	0.000	NS	NS	NS	NS	NS
Docosapent-aenoic Acid 22:5 n-3	1.11 (0.07)	1.39 (0.08)	1.13 (0.04)	1.31 (0.04)	1.34 (0.06)	1.43 (0.06)	1.36 (0.46)	1.52 (0.05)	0.000	0.000	NS	NS	NS	NS	NS
Docosahex-aenoic Acid 22:6n-3	7.36 (0.53)	6.62 (0.58)	6.61 (0.32)	7.07 (0.31)	3.00 (0.46)	3.76 (0.43)	3.15 (0.35)	3.93 (0.39)	NS	0.000	NS	NS	NS	NS	NS
Docosapent-aenoic Acid 22:5 n-6	3.07 (0.29)	2.41 (0.32)	3.24 (0.18)	2.37 (0.17)	3.00 (0.25)	2.80 (0.24)	3.00 (0.19)	2.90 (0.21)	0.005	NS	NS	NS	NS	NS	NS

Data presented as mean ± SEM (N=87)
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test.
NS data not significantly different

the affected 3 week animals with the diseased animals having a 12 % lower AA composition than normal animals (24.32 ● 0.87 vs 27.77 ± 1.05 %, P<0.05).

In the liver, an overall diet effect occurred, with the animals fed soy protein having a 9 % higher AA composition when compared to the casein fed animals (20.08 ± 0.50 vs 18.74 ± 0.50 %, P=0.000) (Table 8). There was an overall time effect in the liver with AA composition lowering over time from 20.23 ± 0.53 % at 1 week to 18.60 ± 1.17 % after 3 weeks of feeding (P=0.000).

4.4.3.3 Docosapentaenoic Acid (DPA) 22:5n-3

Renal DPA n-3 was detected in trace amounts and was not statistically analyzed for differences. Significant levels were found in the liver and demonstrated overall diet and time effects (Table 8). DPA levels were 1.15 times higher in the animals fed soy protein when compared to the casein fed animals (1.41 ● 0.06 vs 1.24 ± 0.06 %) (P=0.000) and DPA levels were 1.15 times higher after 3 weeks of feeding (1.41 ± 0.06 %) when compared to those animals after 1 week of feeding (1.24 ± 0.06) (P=0.000).

4.4.3.4 Docosahexaenoic Acid (DHA) 22:6 n-3

There was no effect of soy feeding or an effect of disease status on DHA composition in both renal and hepatic tissues. DHA composition decreased over time with the 1 week fed animals having a 62 % higher DHA composition than the 3 week fed animals in the kidney (2.75 ± 0.11 % vs 1.70 ± 0.11 %, P=0.000) as seen in Table 7. A more dramatic decrease was found in the liver, with double the amount of DHA found after 1 week of feeding with the levels dropping from 6.92 ± 0.4% to 3.45 ± 0.4% at 3 weeks of feeding (P=0.000) (Table 8).

4.4.3.5 Docosapentaenoic Acid (DPA) 22:5 n-6

The identification of this peak is not confirmed due to unavailability of appropriate commercial standards and lack of proper instrumentation for proper identification (ex. GC mass spectrophotometer). Research examining fatty acids in piglets have examined 22:5n-6 in kidneys and livers and the levels of the fatty acid that peaked at 47 minutes was similar to the reported 22:5 n-6 found in the piglets and rats (Arbuckle et al, 1994; Bourre et al, 1989). Based on the above literature and the elimination of other major fatty acids based on various standards, this peak is considered to be 22:5 n-6. This fatty acid was detected in renal tissue but at levels lower than 1%. In the liver, an overall diet effect was observed (Table 8). DPA n-6 in the animals fed soy protein had a lower 22:5 n-6 level than the casein fed animals (2.62 ± 0.24 vs 3.08 ● 0.23, P=0.005). Levels did not change over time.

4.5 Fatty Acid Ratios

4.5.1 16:1 n-7/16:0 n-7

In the kidney, a time by disease interaction was detected in the 16:1/16:0 ratio (P=0.039). A difference was found in the 3 week fed animals only, with the normal animals having a lower ratio (0.042 ● 0.008) than the diseased animals (0.068 ± 0.006) (P<0.05). In the hepatic tissue, only a diet effect was detected with the animals fed soy protein having a 51 % lower ratio than the animals fed casein protein (0.035 ± 0.003 vs 0.072 ± 0.003, P=0.000).

Table 9 Kidney fatty acid ratios of normal and affected Han:SPRD-*cy* rats fed casein or soy protein for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
Ratios	Normal		Affected		Normal		Affected								
	Casein	Soy	Casein	Soy	Casein	Soy	Casein	Soy	Diet	Time	PKD	Time x PKD	Diet x PKD	Time x Diet	Time x Diet x PKD
16:1/16:0 n-7	0.047 [ab] (0.013)	0.052 [ab] (0.014)	0.052 [ab] (0.008)	0.035 [a] (0.007)	0.048 [ab] (0.011)	0.036 [ab] (0.011)	0.068 [b] (0.008)	0.069 [ab] (0.010)	NS	NS	NS	0.039	NS	NS	NS
18:1/18:0 n-9	0.58 (0.12)	0.77 (0.13)	0.71 (0.08)	0.56 (0.07)	0.56 (0.10)	0.49 (0.10)	0.83 (0.08)	0.57 (0.09)	NS	NS	NS	NS	NS	NS	NS
20:4/18:2 n-6	1.90 [ab] (0.16)	1.36 [a] (0.18)	1.95 [b] (0.10)	1.59 [a] (0.09)	2.21 [b] (0.14)	1.89 [ab] (0.13)	1.81 [ab] (0.11)	1.74 [ab] (0.12)	0.001	0.028	NS	0.029	NS	NS	NS

Data presented as mean ± SEM
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test
NS data not significantly different.

Table 10 Liver fatty acid ratios of normal and affected Han:SPRD-*cy* rats fed casein or soy protein for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
Ratios	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
16:1/16:0 n-7	0.071 (0.007)	0.029 (0.008)	0.072 (0.004)	0.034 (0.004)	0.079 (0.006)	0.042 (0.006)	0.065 (0.005)	0.036 (0.005)	0.000	NS	NS	NS	NS	NS	NS
18:1/18:0 n-9	0.69 (0.08)	0.53 (0.09)	0.76 (0.05)	0.54 (0.05)	1.24 (0.07)	0.88 (0.07)	1.11 (0.06)	0.78 (0.06)	0.000	0.000	NS	NS	NS	NS	NS
20:4/18:2 n-6	1.43 [a] (0.06)	1.20 [b] (0.06)	1.44 [a] (0.03)	1.20 [b] (0.03)	0.87 [c] (0.05)	0.90 [c] (0.05)	0.88 [c] (0.04)	0.95 [c] (0.04)	0.003	0.000	NS	NS	NS	0.000	NS
22:5/20:4 n-6	0.15 (0.02)	0.12 (0.02)	0.17 (0.01)	0.12 (0.01)	0.17 (0.01)	0.15 (0.01)	0.17 (0.01)	0.15 (0.01)	0.000	0.014	NS	NS	NS	NS	NS
22:6/22:5 n-3	6.65 [a] (0.50)	4.84 [a] (0.55)	5.87 [a] (0.31)	5.55 [a] (0.29)	2.20 [b] (0.44)	2.53 [b] (0.41)	2.37 [b] (0.33)	2.58 [b] (0.37)	NS	0.000	NS	NS	NS	0.024	NS

Data presented as mean ± SEM
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test
NS data not significantly different.

4.5.2 18:1 n-9/18:0 n-9

There was no significant differences detected in the kidney 18:1/18:0 ratio. In the liver, an overall diet and time effect occurred. With respect to diet, the soy fed animals had a 32 % lower ratio than the casein fed animals (0.129 ± 0.006 vs 0.189 ± 0.006, P=0.000). Over time, the 3 week animals had a 54 % higher ratio when compared to the 1 week fed animals (0.193 ● 0.006 vs 0.125 ± 0.007, P=0.000).

4.5.3 20:4 n-6/18:2 n-6

Although a time by disease interaction did occur in the kidney, there was no significant difference found between diseased and normal animals or between 1 and 3 week fed animals. An overall diet effect was detected in the kidney, with the animals fed soy protein demonstrating a 16 % lower 20:4/18:2 ratio than the casein fed animals (1.65 ± 0.07 vs 1.97 ± 0.07, P=0.001)

In the liver, a time by diet interaction occurred (P=0.000). After 1 week of feeding, animals fed soy protein had a 16 % lower ratio in the normal animals and a 17 % lower ratio in the diseased animals when compared to their casein fed counterparts (P<0.05). Over time, all groups in the 1 week feeding trial had higher ratios than their 3 week fed counterparts P<0.05). There were no disease effects detected.

4.5.4 22:5 n-6/20:4 n-6

This ratio was only calculated in the liver fatty acids. An overall diet and time effect was detected. In respect to diet, the soy fed animals had a 21 % lower ratio than the casein fed animals (0.131 ● 0.006 vs 0.166 ● 0.006, P=0.000). Over time, the 3 week fed

animals had a higher ratio than those fed for 1 week (0.159 ± 0.006 vs 0.137 ● 0.006, P=0.014).

4.5.5 22:6 n-3/22:5 n-3

This ratio was calculated only in the liver fatty acids where a time by diet interaction occurred (P=0.024). There were no differences found in the respect to diet but there was a time effect found in each group with the 1 week fed animals having a higher ratio when compared to their 3 week fed counterparts (P<0.05).

4.6 Prostaglandin E_2 (PGE_2)

Prostaglandin E_2 was measured through ex vivo release and has been expressed in three ways; as nmol/g of solid kidney tissue (total kidney weight – cyst volume), nmol/kidney (Figure 13); and the amount of PGE_2 per gram of tissue (nmol/g tissue). The nmol/g of solid tissue resembled the whole kidney concentration and therefore only the whole kidney concentration will be explained in detail. Numbers for all data can be found in Table 9.

A time by disease interaction occurred in the whole kidney calculation. The normal animals remained statistically equivalent over time, while the diseased animals demonstrated a dramatic increase in PGE_2 levels, more than doubling the content (2.12 times) from 0.58 ± 0.07 nmol/kidney at 1 week vs 1.24 ● 0.08 nmol/kidney at 3 weeks (P<0.05). There was no significant difference between diseased and normal after either 1 or 3 weeks of feeding or between casein and soy fed animals in PGE_2 release per kidney.

A diet by disease effect was detected in the calculation of PGE_2 per gram of renal tissue (P=0.021). There were no differences between normal and diseased fed animals

with respect to diet (Figure 14). However, the diseased animals fed casein proteins exhibited a 45 % lower PGE_2 level when compared to the normal animals fed casein protein (0.72 ± 0.13 vs 1.32 ± 0.13 μmol/g, $P<0.05$). There was no difference detected between normal and diseased animals fed soy protein. There was also no effect of time on the amount of PGE_2 per gram of tissue.

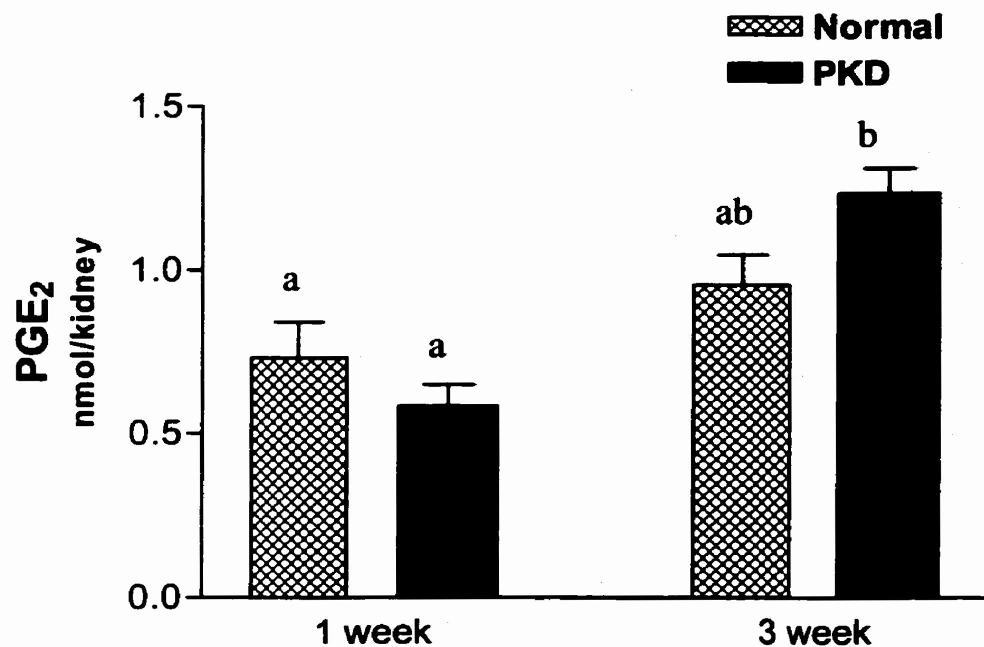

Figure 13 Renal PGE_2 calculated per kidney in diseased and normal animals fed for 1 or 3 weeks. Data are expressed as mean ± SEM (N=87). Columns with different letters indicate a significant difference at $P<0.05$ using Tukey's b post hoc test. Time by disease interaction; $P=0.017$.

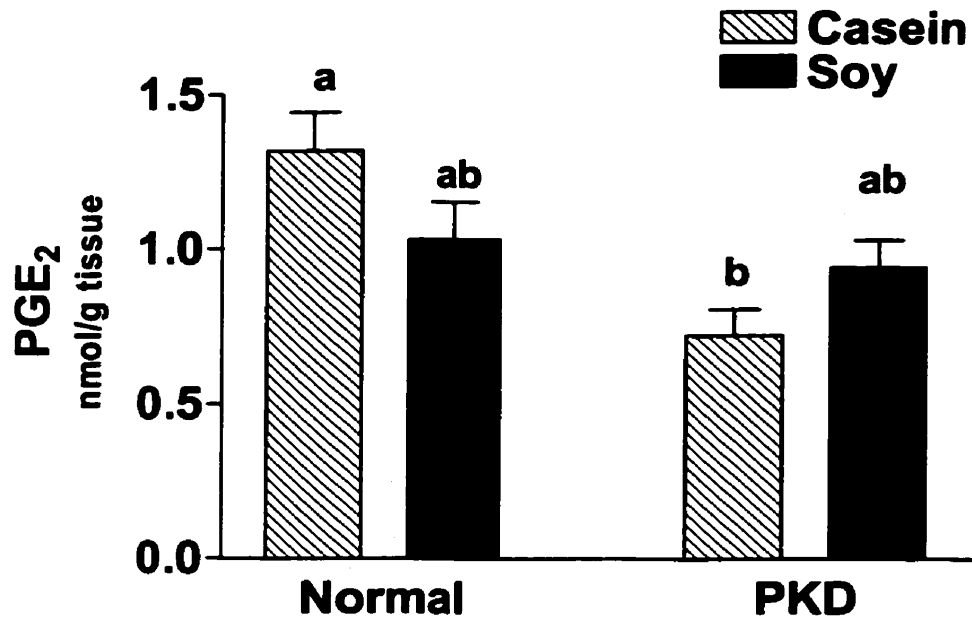

Figure 14 Renal PGE_2 calculated per gram of tissue in diseased and normal animals fed soy or casein protein. Data are expressed as mean ± SEM (N=87). Columns with different letters indicate a significant difference at $P<0.05$ using Tukey's b post hoc test. Time by disease interaction; P=0.021.

Table 11 Prostaglandin E_2 ex vivo release from renal tissue from normal or Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
PGE_2 (nmol/ kidney)	0.81 ^{ab} (0.16)	0.65 ^{ac} (0.16)	0.51 ^{a} (0.10)	0.65 ^{ac} (0.09)	1.06 ^{bc} (0.14)	0.84 ^{ab} (0.13)	1.26 ^{b} (0.10)	1.21 ^{b} (0.12)	NS	0.000	NS	0.017	NS	NS	NS
PGE_2 (nmol/ g)	1.45 ^{a} (0.19)	1.09 ^{ab} (0.19)	0.65 ^{b} (0.12)	0.85 ^{ab} (0.11)	1.20 ^{ab} (0.17)	0.98 ^{ab} (0.16)	0.79 ^{b} (0.13)	1.03 ^{ab} (0.14)	NS	NS	0.002	NS	0.021	NS	NS
PGE_2 (nmol/g of solid tissue)	0.81 ^{ab} (0.16)	0.65 ^{a} (0.16)	0.50 ^{a} (0.09)	0.63 ^{a} (0.09)	1.06 ^{ab} (0.13)	0.84 ^{ab} (0.13)	1.22 ^{b} (0.10)	1.18 ^{b} (0.11)	NS	0.000	NS	0.020	NS	NS	NS

Data presented as mean ± SEM
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test
NS data not significantly different.

5 Discussion

PKD is the most common renal genetic disease, causing ESRD in around 50 % of its patients by the age of 60. Although there is no cure for this disease, dietary intervention, particularly with soy protein, has been shown to attenuate the progression of this disease (Williams et al, 1987; Ogborn et al, 1998; Tomobe et al, 1998; Aukema et al, 1999; Aukema et al, 2001).

The exact mechanism of how soy alters renal disease remains unclear but soy protein does alter renal and hepatic fatty acid composition (Ogborn et al, 2000) and hepatic Δ6 desaturase enzyme, a key enzyme in fatty acid metabolism (Lindholm and Eklund, 1991).

Unfortunately the majority of studies conducted on soy protein have initiated a dietary intervention upon renal insufficiency and not during early renal diseases. Of those studies starting during the early stages of the disease, feeding trials were conducted for long periods, making it impossible to determine the early influences of soy on cyst development. The current short term feeding trial during the early stages of cyst development have provided insight into the effects of soy protein on disease progression and cyst development.

The **hypotheses** of this study were that soy protein feeding to weanling Han:SPRD-*cy* rats in early renal disease would maintain renal function and delay cyst development and disease progression after 1 and 3 weeks of feeding. Soy protein would alter polyunsaturated fatty acid metabolism and modify fatty acid status in both renal and hepatic tissues and would alter prostaglandin E_2 (PGE_2) production.

The **objectives** of this research were to determine whether dietary soy protein, after as early as 1 to 3 weeks of feeding in weanling Han:SPRD-*cy* rats would alter:

1. cyst development and disease progression of chronic renal disease
2. hepatic and renal fatty acid composition as seen previously after 6 weeks of feeding
3. eicosanoid metabolism by quantifying renal PGE_2 ex vivo release

5.1 Animal Growth

An increase in mortality and poor growth during soy feeding intervention studies of animals with renal disease has occurred previously (Huang et al, 1986, Ogborn and Sareen, 1995). All diseased animals survived their feeding trial and grew on their respective diets as indicated by an increased body weight, femur length and femur weight from 1 to 3 weeks of feeding. The prevalence of disease was clear, even after 1 week of feeding as seen by an increase in kidney weight and evidence of renal cysts and renal fibrosis content. This is consistent with previous research on this animal model (Kaspareit-Rittinghausen et al, 1991).

Casein fed animals were pair fed against the soy fed animals. However, the average daily intake of the casein fed animals was lower than the soy fed animals. It is unlikely that the slightly higher protein intake in soy fed animals had an effect on disease progression as soy protein has been shown to have similar effects at both normal (24% protein) and low protein (12% protein) intakes (Williams et al, 1987). Other studies of soy feeding in PKD animals show that at equivalent intakes, soy protein compared to casein feeding reduces cyst development (Tomobe et al, 1998; Aukema et al, 1999; Aukema et al, 2001)

5.2 Disease Progression

Soy protein feeding demonstrated a beneficial effect on disease progression as early as 1week of feeding as indicated by a reduction in renal fibrosis volume. After 3 weeks, rats fed soy protein when compared to animals fed casein protein had less cyst volume area and less total kidney weight, which is consistent with previous studies after longer term soy protein feeding (Ogborn et al, 1998; Aukema et al, 1999; Aukema et al, 2001). This implies that after 3 weeks of feeding, soy protein has a protective effect against disease progression by limiting cyst growth and lowering total kidney weight. A lower kidney weight suggests less cyst growth and fluid accumulation and an overall reduction in disease progression.

Soy protein did not influence serum triglyceride or serum cholesterol levels, although many studies have demonstrated a hypocholesterolemic effect when feeding soy protein (Anderson et al, 1995; Sirtori et al, 1977; Kanazawa et al, 1995). Anderson et al (1995), demonstrated a 0.60 mmol/L decrease in human cholesterol levels and Sirtori et al (1977), reported a 14 % decrease in cholesterol levels after 2 weeks of feeding soy and a 21 % decrease after 3 weeks of feeding soy in humans. This lack of response to soy could be attributed to the short duration of the feeding trials, not enabling sufficient time to lower serum levels. In addition, rats are extremely efficient at producing cholesterol, making it difficult to lower cholesterol through dietary measures. These findings are consistent with a previous study on the Han:SPRD-*cy* rat model that did not demonstrate a hypocholesterolemic effect after 6 weeks of feeding soy protein (Ogborn et al, 2000).

Renal function remained normal in the diseased animals fed soy protein for 3 weeks of feeding as indicated by similar levels of creatinine clearance in diseased

animals fed soy when compared to normal animals at 3 weeks. In contrast, casein fed diseased animals had a remarkable reduction in creatinine clearance when compared to soy fed diseased animals. Casein is known to cause hyperfiltration and is thought to contribute to the progression of renal disease via hydraulic injury to the glomeruli over time. Creatinine clearance in casein fed animals initially increases due to hyperfiltration but gradually decreases as renal tissue is destroyed and GFR decreases. This may account for the reduction seen in casein fed diseased animals while preserving renal function in soy fed animals. Soy protein seems to prevent a reduction in creatinine clearance, indicating that soy feeding in diseased animals has a beneficial effect on preserving renal function.

5.3 Fatty Acids

5.3.1 Diet

In addition to altering disease progression, soy protein also influences PUFA metabolism and tissue fatty acid composition. In the kidney, soy protein feeding decreased palmitic acid in both the 1 and 3 week feeding trials and LA composition after 1 week of feeding only. Ogborn and colleagues (2000), reported no differences between animals fed casein and soy protein in palmitic acid, but did demonstrate an increase in LA in soy fed animals. This study also demonstrated changes in vaccenic acid, LNA and lignoceric acid that were not seen in the current study. The discrepancies between the studies may be due to the age difference at time of measurement and different degrees of renal insufficiency.

Soy protein feeding had a more dramatic effect at altering fatty acid composition in the liver. Soy protein feeding decreased palmitic acid, palmitoleic acid, oleic acid, vaccenic acid and DPA n-6 when compared to casein fed animals and increased stearic acid and AA. Soy feeding also increased LA composition after 1 week of feeding when compared to casein fed animals but not after 3 weeks. These findings are not consistent with Ogborn and colleagues (2000) with differences lying between all fatty acids with the exception of LA, which after 1 week in this study was increased by soy feeding and was also increased by soy feeding in the 6 week study. Again, these differences may be due to age difference and different stages of renal sufficiency.

These variations in liver fatty acids between casein and soy protein feeding suggest an alteration in hepatic desaturase activity. Hepatocytes have the ability to metabolize fatty acids into longer and more unsaturated fatty acids using the desaturase and elongase enzymes. The enzyme Δ9 desaturase is responsible for the conversion of palmitic acid to palmitoleic acid and stearic acid to oleic acid, while LA is converted to AA by the Δ5 and Δ6 desaturase enzymes and elongase enzymes. Δ4 desaturase and elongase enzymes are used in the conversion on AA to 22:5n-6 and may also be used in the conversion of 22:5n-3 to 22:6 n-3 (Jones and Kubow, 1999), although the conversion of 22:5n-3 to 22:6 n-3 may be due to the enzymes Δ6 desaturase and elongase and β-oxidation (Figure 1)(Sprecher et al, 1995).

The ratios of product to substrate provide insight into possible alterations in desaturase activity. In the liver, both 16:1/16:0 n-7 and 18:1/18:0 n-9 ratios are lowered in the soy fed animals when compared to their casein counterparts, suggesting a reduction in Δ9 desaturase activity. When examining the AA/LA ratio, soy lowers this ratio after 1

week of feeding only and not after 3 weeks of feeding, suggesting that soy has a possible early influence of soy protein on Δ6 and Δ5 desaturase and/or elongase activity. Δ4 desaturase activity and/or elongase could also be reduced by soy protein feeding as demonstrated by a lower 22:5/20:4 n-6 ratio than the casein fed animals although the 22:6/22:5 n-3 ratio was not altered by soy.

It has been documented that soy protein alters hepatic Δ6 desaturase activity (Huang et al, 1986, Lindholm and Eklund; 1991, Koba et al, 1993) and a reduction in Δ9 desaturase has also been implied during soy protein feeding (Lindholm & Eklund, 1991)

Soy could alter desaturase activity through several mechanisms. Koba and colleagues (1993), discovered an association between membrane fluidity and the activity of the membrane bound Δ6 desaturase enzyme. Membrane fluidity is altered by dietary protein, in particular by soy protein, by lowering the cholesterol to phospholipid ratio in the cell membrane. As membrane fluidity increases, there is a decrease in Δ6 desaturase activity. The hypothetical pathway is that soy protein lowers the cholesterol to phospholipid ratio, which may cause an increase in membrane fluidity and a decrease in Δ6 desaturase activity (Sugano & Koba, 1993). If this is the mechanism of how soy alters desaturase activity then all desaturase enzymes could be influenced by soy protein feeding as all desaturase enzymes are bound to the cellular membrane.

However, the effect of soy protein on Δ6 desaturase activity may also be due to its altered amino acid composition. The suppressive effect of soy protein on Δ6 desaturase activity may be exerted, in part, through the release of hormones such as glucagon. Soy protein has a high arginine content and has been shown to induce a low insulin/glucagon

ratio (Sanchez and Hubbard, 1991). One of the physiological activities of a high glucagon level is to reduce liver microsomal Δ6-desaturase activity (Jones & Kubow, 1999).

Soy protein clearly alters the fatty acid status in hepatic tissues possibly through a reduction in desaturase and/or elongase activity. However, ratios in renal tissue only demonstrated a diet effect in 20:4/18:2 n-6 suggesting that there is less effect of soy protein on renal desaturase and elongase activity. This limited influence of soy protein on renal desaturase enzymes could be due to a reduced PUFA metabolism in the kidney when compared to the liver. The liver is the predominant site of PUFA metabolism and although the kidney does metabolize fatty acids, it does so at a much lower rate than the liver (Kopple and Jones, 1999). Therefore, this reduced influence of soy protein on renal desaturase enzymes could be due to the fact that there is less PUFA metabolism in the kidney when compared to the liver.

5.3.2 Time and Disease

With respect to time, the liver fatty acids stearic acid, AA and DHA had a overall lower composition after 3 weeks when compared to the 1 week levels and oleic acid, vaccenic acid and DPA n-3 had an overall higher level after 3 weeks of feeding. LA was higher after 3 weeks of feeding in the animals fed casein protein only. In the kidney, DHA levels decreased in all groups, stearic acid decreased over time in the normal animals fed soy protein and AA composition increased over time in the normal animals.

These alterations in the fatty acid compositions in the liver over time are likely due to differences in fatty acid requirements for growth and development as these animals are at different levels of maturation. The energy requirements for growing

animals are higher than adults due to a higher resting metabolic rate and special needs for growth and development (Pahl et al, 1990).

Each class of fatty acid is involved in specific metabolic reactions during growth and development. Medium and saturated long chain fatty acids are a good source of energy, polyunsaturated fatty acids are involved in metabolic regulation and very long chain fatty acids are important structural components of membranes (Giovannini et al, 1991). The elevations and reductions of fatty acids are probably due to the fluctuations in tissue growth and development. The rate of growth in animals is not linear or continuous and fatty acid requirements and utilization can change over time.

There are specific nutrients such as AA and DHA that are essential for growth and development. Both AA and DHA composition fell over time in the hepatic tissue. This reduction in AA could be a result of an increased requirement for this fatty acid for growth, thus leaving less available to be stored in the cells. The same is true for DHA which is required for neural and retinal development (Innis, 1991). An increased requirement for these fatty acids, would reduce the amount stored in hepatic cells and, depending on the requirement may also limit the storage of these fatty acids in other tissues such as the kidney.

In renal tissue, DHA is also reduced over time, suggesting there is less need for DHA as the tissue matures and less DHA is produced. However, when examining the effects of time on renal AA levels, the effect of disease also becomes a factor. Over time, the normal animals had an increase of AA while the diseased animals remained constant. No differences were detected between diseased and normal animals after 1 week of feeding but after 3 weeks, the diseased animals had a lower AA composition than the

normal animals. These alterations over time in addition to the difference found after 3 weeks of feeding in the diseased animals suggests that the disease state alters AA composition. It suggests that normal animals have a reduced requirement of AA over time allowing for AA levels to increase in cellular membranes. However, diseased animals have less stored AA suggesting an increased usage of this particular fatty acid. There are many possibilities that may explain this lower AA composition in diseased animals in renal tissues.

The reduction could be due to an increase in the liberation of membrane bound AA in diseased animals by an increase in the activity of the PLA_2 enzymes. This increase in liberation could be linked to an increase in eicosanoid production, which is commonly seen in chronic diseases. Research has demonstrated an increase in COX-2 activity in the renal ablation model (Sanchez et al, 1999) while studies on the Han:SPRD-*cy* rat model and the *pcy* mouse model of PKD have demonstrated an increase in COX-1 and PLA_2 enzymes (Aukema and Jiang, 2000) in diseased animals. Both of these studies support the possibility that the reduction in AA is due to an increase in eicosanoid production.

It has been shown that as the disease progresses, there is less functional capacity of the kidney due to an increase in cyst volume and renal fibrosis volume. A lower functional capacity in diseased tissue could reduce the kidney cells' ability to produce AA from its precursor LA, there by reducing the amount of AA. The kidney does not produce its entire AA amount and imports AA from hepatic cells. It is also possible that a reduction in functional capacity could reduce the ability of the kidney to incorporate AA imported from hepatic tissues. Further research is required to determine the effect of disease and its effects on the production and incorporation of long chain PUFAs.

5.4 Prostaglandin Production

The concept that a decrease in renal AA in diseased animals is due to altered eicosanoid production is supported by an increase in renal PGE_2 release over time in diseased animals when examining whole kidney PGE_2 release. In diseased kidneys, PGE_2 levels doubled from 1 week to 3 weeks of feeding in the diseased animals, while in the normal animals PGE_2 release remained consistent over time suggesting that as renal disease progresses there is an increase in PGE_2 production. Weiler and colleagues (unpublished) demonstrated a higher PGE_2 release in renal tissue in diseased Han:SPRD-*cy* rats that were fed casein for a 6 week period and were older at time of measurement. PGE_2 is also elevated in other animal models of chronic renal injury (Peck et al, 1989, Stahl et al, 1987). As previously mentioned, there is an increase in the COX-1 and PLA_2 enzymes in the Han:SPRD-*cy* rat (Aukema & Jiang, 2000) which could account for this increase in PGE_2 levels in diseased animals. Unfortunately, it is hard to apply this directly to eicosanoid production, as the measure of PGE_2 release may not be representative of PGE_2 production or indicate where the PGE_2 is produced. PGE_2 could be produced by only healthy tissue or be produced by cyst epithelia and stored in cyst fluid.

With respect to the amount of PGE_2 per gram of tissue, only the animals fed casein demonstrated an effect, with the normal animals fed casein having more PGE_2 release than the PKD animals, while both normal and diseased soy fed animals remained similar. This lower PGE_2 release per gram of tissue in diseased animals fed casein suggests that there is a reduction in the functional capacity of the renal tissue when compared to normal animals fed casein. This implication of lower functional capacity per gram of tissue is supported by a lower creatinine clearance and higher cyst volume and

renal fibrosis volume in this group after 3 weeks of feeding. An increase in cysts and fibrosis would result in less healthy tissue per gram of tissue, leaving less tissue that is capable of producing PGE_2. It is likely that the effect of diet on PGE_2 release is a result of the disease and is not a causative effect on disease progression.

Although this study did not provide insight into the effects of soy protein on PGE_2 levels, future studies examining the long term effect of soy protein feeding on PGE_2 metabolism in this model are needed to determine if soy protein would reduce PGE_2 formation given a longer feeding trial.

5.5 Possible Effects of Soy on Renal Disease Progression

The benefits of soy protein are likely to be multifactorial. In this research, soy protein clearly altered disease progression after 1 and 3 weeks of feeding as indicated by reduced cyst and fibrosis volume and the preservation of creatinine clearance in diseased animals fed soy. Although it is possible that fatty acids play a role in the attenuation of renal disease, it is unlikely that soy protein effects on PGE_2 are the main mechanism of action as there was no significant difference in PGE_2 release between the soy and casein fed animals.

There are other possible mechanisms of how soy protein alters disease progression and renal function. Isoflavonoids have been implicated but it is not clear whether these benefits are due to isoflavonoids (genistein and daidzein) or some other component of soy. Even within the context of isoflavonoids, there are a number of possible mechanisms that could be influenced. Isoflavonoids are phytoestrogenic and have antiproliferative properties (Setchell, 1998). Phytoestrogens act as weak estrogens

and compete with estradiol (endogenous hormone) for binding to intranuclear estrogen receptors that modulate gene transcription (Martin et al, 1978). Isoflavones have been shown to reduce cell proliferation in many cell lines through their action with estrogen receptors (Hirano et al, 1990, Yanajigara et al, 1993) and could have implications of the rapid cell growth seen in cyst epithelia.

Phytoestrogens may exert their antiproliferative actions through non estrogen receptor mediated mechanisms. Genistein can inhibit protein tyrosine kinase (Akiyama et al, 1987), DNA typoisomerases I and II (Markovits et al, 1989) and ribosomal S6 kinase (Linassier et al, 1990). Protein tyrosine kinase is associated with cellular receptors for several growth factors (ex. EGF) and transcription factors important in signaling molecules involved in cell inflammation and oxidative stress (Lan et al, 1994). In both animal and humans forms of PKD, there is an increase in proliferation of tubular cells in the cystic epithelium (Floege & Grone, 1995, Grantham, 1996, Cowley et al, 1993) that has been associated with the cellular expression of EGF, a mediator of cell proliferation and fibrogenesis. Studies by Avner and colleagues (1999) have demonstrated that cystic epithelial cells have abnormalities in the amount (too many) and location (apical surface rather than basal surface) of the EGF receptor. These alterations, in conjunction with high EGF, can increase cellular proliferation. Isoflavones may target this proliferative component of PKD and slow the enlargement of cysts by inhibiting proliferative mediators such as EGF.

Genistein may also exert beneficial effects through its antioxidative action. Hyperlipidemia is a common manifestation in renal disease and accumulation of oxidized lipoprotein in the kidney has been suggested to accelerate glomerular and

tubulointerstitial damage (Schmitz et al, 1989). It is suggested that genistein may limit lipid induced renal injury by reducing plasma lipoprotein concentrations or reducing lipid peroxidation in injured cells through their antioxidative actions (Anthony et al, 1998, Wei et al, 1995). However, this research did not find a diet effect of soy on triglyceride and cholesterol levels, possibly due to the short duration of the feeding trials and the young age of the animals. Longer studies may allow soy protein and the effects of genistein to demonstrate a hypocholesterolemic and antioxidative effect.

It is also important to note that previous studies examining the effect of genistein alone did not attenuate renal disease or reduce cyst development (Tomobe et al, 1998, Ogborn et al, unpublished). It is possible that genistein alone has little effect at reducing cyst development but in combination with other effects of soy protein such as alterations in fatty acid, may have a synergistic effect on altering renal disease progression. Further studies are required to determine this possibility.

The amino acid composition of soy may play a role in attenuating disease progression in other ways than altering desaturase activity. Soy protein contains a higher percentage of arginine, around 7.5%, while casein has only 3.7% (Harlan Teklad, 2001). However, data to date are not conclusive with experiments on arginine supplementation and restriction both displaying beneficial effects on renal tissue. Arginine is a substrate for at least three products involved in tissue injury and fibrosis. It is a precursor for proline, a major component of the collagen that results in fibrosis. It is also a precursor for polyamines, compounds involved with the proliferative responses found in many renal diseases. Arginine can also be converted to nitric oxide (NO) and when NO is produced in large quantities it is involved in tissue injury (as reviewed by Peters and

Noble, 1996). Therefore restriction in dietary arginine could reduce tissue damage and injury by reducing proline, polyamine and NO production.

Conversely, arginine supplementation on the kidney may improve renal blood flow and GFR and reduce proteinuria in rat renal nephrosis (Reyes et al, 1994). It appears that nitric oxide produced in smaller amounts is a potent vasodilator, which has been shown to reduce intraglomerular pressure by counteracting the vasoconstrictive actions of the renin-angiotensin system (Peters and Noble, 1996). Further research in this area is required.

5.6 Summary

In summary, disease progression was attenuated after as early as 1 week of feeding soy protein as indicated by a lower renal fibrosis volume. By 3 weeks of feeding, soy protein reduced renal cyst development and preserved renal function and lowered kidney weights. Soy protein feeding alters renal and hepatic fatty acid composition but not PGE_2 release after 1 week of feeding. Although AA derived prostaglandins are commonly found in excess in chronic renal disease, it is unlikely that the effects of soy protein on PGE_2 production is a main contributing factor in early disease as there was no effect of soy protein on PGE_2 release.

This research thesis demonstrates the importance of early dietary intervention on the progression of renal disease. PKD is an infantile disease, with the early stages of cyst development beginning during infancy. Soy protein feeding seems to have a beneficial effect if fed earlier in disease progression, as it seems to target cyst development and fibrosis. Although the exact mechanisms remain under investigation, it is clear that early

dietary intervention of soy protein on post weaning male rats attenuated disease progression.

The hypotheses of this thesis research are accepted with the exception that soy protein did not alter PGE_2 release. It is thought however, that longer feeding trials will demonstrate this part of the hypothesis to be true as soy protein has been shown to alter eicosanoid production in previous studies on diabetic rats (Ikeda and Sugano, 1993).

5.7 Limitations and Strengths

A research limitation was the use of the radioimmunoassay kit for determination of PGE_2 release. Although this method is widely used, the cross reactivity to PGE_1 and PGE_3 is relatively high (70 % and 16 % respectively). Although these PGs are not commonly found in normal renal tissue, it is possible that during renal disease there may be a shift in eicosanoid production from the 2 series of eicosanoids to the 1 or 3 series of prostaglandins. This may result in a skewed PGE_2 amount and the other two PGs being undetected. This could be overcome by using a more extensive method, such as HPLC or GC mass spectrophotometer that would separate the PGs and allow for a more definitive conclusion.

The method of PGE_2 quantification has some limitations. The PGE_2 was quantified by measuring the amount released from renal tissue after a one hour incubation time. A limitation arises in what the amount of PGE_2 represents. During this time of incubation, PGE_2 is released from the tissue and cyst fluid but some PGE_2 could be produced or broken down during this time. A time zero quantification would have given an indication of what levels were already present in the kidney before incubation. This

could be done by obtaining a sample from the homogenized tissue in Hank's Solution prior to incubation.

An increase in the amount of time in the metabolic cages for urine collections would have helped get a more accurate reading of creatinine clearance as the formula used is calculated using total amount of urine per 24 hour period. However, due to ethical protocol, animals are not allowed to be without food and water for more than 6 hours, making a longer collection time unfeasible. Possibly a better method of collection could be used but this method did demonstrate a difference and was consistent with the other parameters such as kidney weight.

The initial sample size was based on previous research done by Ogborn et al, 2000 and an estimated sample size of 6 was sufficient. Upon completion of this study, calculation of minimum sample size for the variances found and using a power of 80% and a type 1 error of 0.05, a sample size of between 8 to 12 animals per group would be necessary for this study. This increase in sample size over Dr Ogborns study is likely due to an increase in variability. This study has added variability due to the growth and development of these animals and the added component of two feeding trials. Future studies examining early intervention would require larger sample sizes.

Another limitation of this research was how the animals were housed. Each animal was placed in a cage with up to four other rats depending on the litter size. This makes it difficult to determine daily food intake per animal and could alter results, as it is impossible to determine if each animal received equivalent amounts of food. Therefore, there could be an effect of food restriction, as each animal did not have equal access to food. However, all animals did grow in this study, indicating that the effects of food

restriction were minimal. Other influences could include cage effects and additional stress to those animals housed by themselves or with only one sibling. Ideally, housing each animal individually would produce less variability and would be recommended in future studies.

A strength of this study is the animal model used. The Han:SPRD-*cy* rat model is an excellent model of PKD and is comparable to PKD in humans in many ways. It develops renal insufficiency early in life making it a good model for early intervention studies. The time course to renal insufficiency is comparable to human PKD. The pathogenesis of this disease and cyst formation in the rat is similar to humans. Lastly, it carries the dominant pattern of gene expression as seen in humans. This animal model is widely used in research and allows for collaboration of research on different aspects of the disease. Research done on one model allows for a greater understanding as a whole and reduces the amount of extrapolation of data from other animal models.

Another strength of this study was that the methods used have been well documented and are reliable. The reliability of these numbers were documented, with replications of all biochemical markers and fatty acids being completed and having reproducible results. In addition, the methods used for both fatty acid analysis and biochemical markers were similar to that of Ogborn et al (2000), allowing for comparison of values.

Lastly, the fact that this was a study examining the early effects of soy using short feeding trials is a study strength. There has been relatively little research done on the early effects of soy on cyst development and disease progression. By implementing the dietary intervention post weaning and for short durations of time, it allowed for an early

examination of how soy protein affects early stages of cyst development and provides insight on how this protein attenuates disease progression.

5.8 Future Studies

Further examination of fatty acids and the role that soy protein plays on altering them should be examined. Currently it is not known if these alterations in renal and hepatic tissues are a consequence of soy protein feeding or in fact part of the mechanism of how soy attenuates renal disease. Examination of fatty acids in combination of other possible mechanisms of actions such as with genistein may provide insight as the exact mechanism of how soy alters renal disease.

The current study examined fatty acid as a percent of total fatty acid composition. It is recommended that future studies examine the total amount of fatty acid in the tissue as well as determine the total percent of fatty acid. This would allow for a more detailed analysis and would allow for comparison to such things as PGs that are often expressed as amount per whole tissue and amount per gram of tissue.

A study similar to this one but with a longer feeding trial, one comparable to Ogborn and colleagues (2000) should be done to examine the role of soy on prostaglandin production after six or eight weeks of soy protein intervention. Previous studies have implicated that prostaglandins are increased in renal disease and play a role in disease progression (Peck1997; Sanchez et al, 1999). Such a study would determine if soy protein feeding alters renal eicosanoid production and may provide further insight into the mechanism of how soy attenuates chronic renal disease and the role of prostaglandins in disease progression.

Future studies should include a more detailed analysis of eicosanoids, possibly using an HPLC or GC-mass spectrophotometer to examine all eicosanoids in the renal tissue. This would reduce cross reactivity and allow for a more detailed examination of all eicosanoids and the effect of disease and diet on their production. It is quite possible that diet or disease results in a shift from one series of eicosanoids to another. A more complete analysis would detect such a change and allow for a more complete analysis of all eicosanoids.

In conjunction with the further examination of eicosanoid production, the next step would be to examine the enzymes responsible for prostaglandin production. The COX enzymes and PLA_2 amounts and activity would be helpful to determine if diet and/or disease alters their activity. This study determined that diseased animals had an increase in PGE_2 over time. It would have been useful determine if it was due to an increase in COX enzyme activity. In addition, it would be interesting to determine if soy does alter PG production and/or shifts to an alternate series of eicosanoids and, if so, if it is mediated through these enzymes.

The role of soy protein and its arginine content has been implied to be a major influence on how soy alters disease progression (Peters and Noble, 1996). It would be beneficial to place tracers or add a radioactive label or isotope to the arginine to examine its affects on the production of NO, polyamines and proline. Similarly, the effect of arginine on desaturase activity and membrane fluidity should be researched further.

Radioactive labels could also be used on dietary LA and LNA to examine the amount of AA and EPA produced in both liver and renal tissues. Currently it is not known whether the kidney produces the majority of renal AA and EPA or whether it is

transported from the liver. By labeling the dietary precursors LA and LNA it would outline the path of these precursors and help to determine whether they are produced in the kidney or if they are imported from the liver.

There have been some amazing discoveries made in soy protein feeding. A study by Lamartiniere and colleagues (1995) determined that feeding rats at days 2, 4, 6 postnatally with genistein reduced the incidence and multiplicity of mammary tumors when compared with vehicle-treated animals. A study similar to this one would be interesting to see if early intervention of soy protein during infancy only can ameliorate renal disease progression. Similarly, a study examining the effects of intrauterine exposure to soy protein by feeding soy protein to mothers and the renal disease progression in the offspring would be of interest. This could have great implications on this disease as it is hereditary and early intervention is key to a long healthy life.

6 APPENDIX

6.1 Appendix A - Staining Protocol

Fixing

1. specimens were fixed in 10% neutral buffered formalin overnight.
2. dehydrated in three changes of alcohol

    95% for 30 minutes

    100% for 30 minutes

    100% for 30 minutes
3. specimens were then cleaned in three changes of xylene for 30 minutes each change
4. impregnated in 2 changes of paraffin was for 1 hour each change (with agitation)
5. sections were cut at 5 microns placed in a 48°C water bath and picked up on a glass slider and place in a 60°C oven for 30 minutes

Dewaxing

1. dewax with three changes of xylal for 3 minutes each change.
2. hydrated to water in 3 changes of 100% alcohol for 30 seconds each change.

Staining – Aniline Blue

1. sections were mordranted in H_2O saturated picric acid for 10 minutes at 60°C
2. washed in d H_2O for 3 minutes
3. sections were placed in phosphomolibdic acid saturated in H_2O for 5 minutes
4. washed for 1 minute then placed in 2.5% aqueous aniline blue for 5 minutes.
5. washed in d H_2O for 10 seconds and dehydrated through 100% alcohol for 2 minutes
6. checked in xylol for 2 minutes and mount slides with cytoseal.

Staining – Hematoxylin and Eosin

1. sections were stained in Harris's hemotoxylin for 5 minutes
2. washed in d H_2O
3. differentiated in 1% HCL in H_2O
4. blued in 2% ammonia water
5. washed in d H_2O
6. placed in eosin for 3 minutes
7. dehydrated in alcohol and mounted with cytoseal.

6.2 Appendix B – Creatinine Clearance

Table 12 Values for the calculation of creatinine clearance corrected for body weight in normal or affected Han:SPRD-*cy* rats fed casein or soy protein based diets for 1 or 3 weeks

	1 week				3 week				P Values and Interactions						
	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Creatinine Clearance * mL/min	0.29^{a} (0.12)	0.26^{a} (0.11)	0.35^{a} (0.07)	0.28^{a} (0.06)	0.89^{bc} (0.10)	1.01^{b} (0.09)	0.66^{c} (0.07)	1.06^{b} (0.08)	NS	0.000	NS	NS	NS	0.009	NS
Urine Creatinine (nmol/L)	11.5 (1.7)	6.4 (1.5)	13.7 (1.0)	8.9 (0.9)	15.1 (1.3)	12.0 (1.2)	15.2 (1.0)	12.7 (1.1)	0.000	0.000	NS	NS	NS	NS	NS
Urine Volume (mL)	0.45^{a} (0.17)	0.66^{ac} (0.15)	0.43^{a} (0.09)	0.61^{a} (0.09)	1.06^{bc} (0.13)	1.91^{b} (0.12)	0.89^{ab} (0.10)	1.82^{b} (0.11)	0.000	0.000	NS	NS	NS	0.000	NS
Serum Creatinine (µmol/L)	47.65^{ab} (4.30)	45.40^{a} (4.30)	48.66^{a} (2.63)	54.54^{ab} (2.55)	48.69^{ab} (3.72)	62.21^{b} (3.72)	55.89^{ab} (2.81)	57.09^{ab} (3.18)	NS	0.004	NS	NS	NS	NS	0.033

* creatinine clearance calculation prior to correction for body weight.

Data presented as mean ± SEM (N=87), NS data not significantly different.
Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test.

6.3 Appendix C – Disease Progression

Table 13 Disease progression indicators from normal or affected Han:SPRD-*cy* rats fed casein or soy protein for 1 or 3 weeks

	1 week				3 week										
Parameter	Normal		Affected		Normal		Affected								
	Casein	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Casein**	**Soy**	**Diet**	**Time**	**PKD**	**Time x PKD**	**Diet x PKD**	**Time x Diet**	**Time x Diet x PKD**
Kidney Weight (grams)	0.59 [a] (0.07)	0.61 [a] (0.07)	0.79 [a] (0.04)	0.78 [a] (0.04)	0.89 [b] (0.06)	0.90 [b] (0.06)	1.66 [c] (0.04)	1.26 [c] (0.05)	0.017	0.000	0.000	0.000	0.004	0.011	0.013
Kidney Weight (g/100g body wt)	0.62 [a] (0.04)	0.62 [a] (0.04)	0.78 [b] (0.02)	0.80 [b] (0.02)	0.46 [c] (0.03)	0.46 [c] (0.03)	0.86 [b] (0.02)	0.63 [a] (0.03)	0.017	0.000	0.000	0.007	0.017	0.003	0.003
Cyst volume (ml/kg body wt)	N/A	N/A	0.21 [a] (0.01)	0.26 [a] (0.01)	N/A	N/A	0.21 [a] (0.02)	0.13 [b] (0.01)	NS	0.001	N/A	N/A	N/A	0.001	N/A
Fibrosis Volume (ml/kg body wt)	N/A	N/A	0.16 (0.01)	0.13 (0.01)	N/A	N/A	0.07 (0.01)	0.04 (0.01)	0.025	0.000	N/A	N/A	N/A	NS	N/A

N/A not applicable as these variables were only measured in diseased animals. NS data not significantly different.
Data presented as mean ± SEM. Groups with different letters in rows are significantly different at $P<0.05$ using Tukey's b post hoc test.

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